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Grant Number DAMD17-96-1-6126

TITLE: Mechanism of c-Src Synergy with the EGFR in Breast Cancer

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REPORT DATE: July 1999

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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DTIC QUALITY INSPECTED 4

19991208 163

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1999	Final (1 Jul 96 - 30 Jun 99)

4. TITLE AND SUBTITLE
Mechanism of c-Src Synergy with the EGFR in Breast Cancer

5. FUNDING NUMBERS
DAMD17-96-1-6126

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200)*

We have shown that inhibition of c-Src in breast tumor cells efficiently blocks tumor formation supporting the hypothesis that it may be an effective therapeutic target. In an effort to isolate a single domain of c-Src to target, we have demonstrated that the SH2 domain and surprisingly the kinase-inactive carboxy terminus of c-Src have inhibitory effects on tumorigenicity and growth of breast tumor cells. Furthermore, we have discovered a mechanism of c-Src synergy with the EGFR and located specific points at which the pathway can be interdicted. Specifically, we have shown that kinase-inactive c-Src is able to inhibit tumorigenicity of the 10T1/2 mouse fibroblast model cells by not phosphorylating the receptor on Tyr 845 in the activation loop of the kinase. The phosphorylation of Tyr 845 is required for EGF, serum, and LPA-induced DNA synthesis through the EGFR. This phosphorylation site in cancer cells presents an appealing target for cancer therapy of tumors that overexpress the EGFR and c-Src, such as breast cancer.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, Receptor Tyrosine Kinases, c-Src,	120
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. 239-18
298-102

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Daniel A Tice 5/24/99

PI - Signature Date

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INTRODUCTION

Cellular Src (c-Src) is frequently overexpressed or activated in human breast cancer. However, the functional significance of this overexpression for the human disease has not been determined. The aims of this grant are to determine the requirement for c-Src in the generation and preservation of a neoplastic phenotype, and to determine a mechanism by which c-Src is required for epidermal growth factor (EGF)-induced mitogenesis and tumorigenesis. Structure/function analysis coupled with biochemical approaches was applied to investigate the role of c-Src in EGF-induced tumorigenicity and overall maintenance of the tumorigenic phenotype. The results demonstrate the requirement for c-Src in tumorigenicity and begin to address its mechanisms of action in promoting tumor cell growth.

BODY

A requirement for Src family members is observed in the progression and maintenance of the tumorigenic phenotype of model tissue culture systems including C3H10T1/2 mouse fibroblasts and human breast tumor cells. In the 10T1/2 cell system, it was previously shown that overexpressed c-Src potentiates EGF-mediated mitogenesis and tumorigenesis. This finding correlates with the association between the two kinases, the appearance of novel phosphorylations on the receptor, and the hyperphosphorylation of receptor substrates. In a structure/function analysis described here, the kinase activity of c-Src, but not the myristylation or Src homology-2 (SH2) functions, was found to be required for synergy with the overexpressed epidermal growth factor receptor (EGFR). Furthermore, c-Src kinase activity was required for phosphorylation of tyrosine 845 on the receptor but not for association of c-Src with the receptor. In transient transfection

assays, not only EGF- but also serum- and lysophosphatidic acid (LPA)-induced DNA synthesis was ablated in a dominant negative fashion by a Y845F mutant of the EGFR, indicating that c-Src-induced phosphorylation of tyrosine 845 is critical for the mitogenic response to both the EGFR and a G protein-coupled receptor (LPA receptor). Unexpectedly, the Y845F mutant EGFR was found to retain its auto-kinase activity and its ability to activate SHC and ERK2 in response to EGF, demonstrating that ERK2-activation is insufficient for EGF-induced mitogenesis.

In the breast tumor cell lines, growth in soft agar or tumor formation in nude mice was inhibited by a Src family kinase inhibitor (PP1) or stable expression of a kinase defective c-Src. A structure/function analysis was conducted to determine which domain(s) of c-Src was responsible for inhibiting tumorigenicity. While the isolated unique and SH3 domains and the amino-terminal half of c-Src had little to no effect, the SH2 domain and surprisingly a kinase inactive form of the carboxy-terminal half of c-Src inhibited DNA synthesis and soft agar growth of MDA-MB-468 breast tumor cells. Furthermore, the carboxy-terminal half of c-Src was found to co-precipitate with an unidentified protein of approximately 95 kilodaltons suggesting a protein interaction function of the carboxy-terminus in addition to its kinase activity. These results implicate the carboxy-terminal half of c-Src as containing two potential therapeutic targets for breast cancer. These findings can be applied towards the development of novel therapeutics for human cancers that overexpress c-Src and/or EGFR.

The third specific aim outlined in the original proposal was not accomplished. The last year was spent writing and finishing experiments for the attached publications and my doctoral dissertation. I successfully completed my PhD on March 30, 1999.

APPENDIX

1.) KEY ACCOMPLISHMENTS:

- Demonstrated that inhibition of c-Src in breast cancer cells blocks tumor formation.
- Demonstrated that expression of either the c-Src SH2 domain or kinase-inactive C-terminus is sufficient to inhibit growth of a breast tumor cell line.
- Showed that phosphorylation of tyrosine 845 on the epidermal growth factor receptor is c-Src-mediated and is required for the mitogenic function of the receptor.

2.) REPORTABLE OUTCOMES:

Degree: PhD, Microbiology, University of Virginia.

Honors: 1998 Michael J Peach outstanding graduate student award

Publications:

Tice, D.A., Karaoli, T., and Parsons, S.J. The Carboxy-Terminus of C-Src Inhibits Breast Tumor Cell Growth by a Kinase-Independent Mechanism. 1999. Submitted.

Tice, D.A., Biscardi, J.S., Nickles, A.L., and Parsons, S.J. Mechanism of c-Src Synergy with the EGF Receptor. Proc.Natl.Acad.Sci. USA. 1999 96: 1415-1420.

Biscardi, J.S., Maa, M-C., Tice, D.A., Cox, M.E., Leu, T-H., and Parsons, S.J. c-Src-mediated Phosphorylation of the Epidermal Growth Factor Receptor on Tyr845 and Tyr1101 Is Associated with Modulation of Receptor Function. J.Biol.Chem. 1999 274: 8335-8343.

Tice, D.A.*, Biscardi, J.S.*, and Parsons, S.J. C-Src, Receptor Tyrosine Kinases, and Cancer. Adv.Cancer Res. 1999 76: 61-119.

* Equal contributions to this work were made by these individuals.

Abstracts:

Tice, D.A., Maa, M-C., and Parsons, S.J. Synergy Between Cellular Src and the Epidermal Growth Factor Receptor in Tumorigenesis. American Association for Cancer Research: Basic and Clinical Aspects of Breast Cancer. 1997; B-40, Poster.

Tice, D.A., Maa, M-C., and Parsons, S.J. Synergy Between Cellular Src and the Epidermal Growth Factor Receptor in Tumorigenesis. Thirteenth Annual International Meeting on Oncogenes. 1997; 45, Poster.

Tice, D.A., Maa, M-C., and Parsons, S.J. Synergy Between Cellular Src and the Epidermal Growth Factor Receptor in Tumorigenesis. Era of Hope: The Department of Defense Breast Cancer Research Program Meeting. 1997; 277-N, Poster.

Tice, D.A., Biscardi, J.S., Nickles, A.L., and Parsons, S.J. Mechanism of c-Src Synergy with the EGF Receptor. FASEB: Receptors and Signal Transduction. 1998; 32, Poster.

Tice, D.A., Karaoli, T., and Parsons, S.J. The Carboxy-terminus of c-Src inhibits breast tumor cell growth by a kinase-independent mechanism. Fifteenth annual meeting on Oncogenes. 1999.

Oral Presentations:

1998 Fourteenth Annual International Meeting on Oncogenes; San Diego, CA.

1998 Virginia American Cancer Society Meeting; Blacksburg, VA.

1996 Virginia American Society for Microbiology; Norfolk, VA.

Post-Doctoral Opportunities Awarded:

- Genentech (Dr. Paul Polakis)
- Bayer (Dr. John Murphy)
- University of Colorado (Dr. Gary Johnson)
- Harvard University (Dr. Philip Leder)
- Fred Hutchinson Cancer Center (Dr. Jonathon Cooper)
- University of Virginia (Dr. Leland Chung)

I am currently undecided.

Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 1415–1420, February 1999
Cell Biology

Mechanism of biological synergy between cellular Src and epidermal growth factor receptor

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Edited by George F. Vande Woude, National Cancer Institute, Frederick, MD, and approved December 18, 1998 (received for review April 27, 1998)

ABSTRACT **Overexpression of both cellular Src (c-Src) and the epidermal growth factor receptor (EGFR) occurs in many of the same human tumors, suggesting that they may functionally interact and contribute to the progression of cancer. Indeed, in murine fibroblasts, overexpression of c-Src has been shown to potentiate the mitogenic and tumorigenic capacity of the overexpressed EGFR. Potentiation correlated with the ability of c-Src to physically associate with the activated EGFR and the appearance of two unique *in vivo* phosphorylations on the receptor (Tyr-845 and Tyr-1101). Using stable cell lines of C3H10T½ murine fibroblasts that contain kinase-deficient (K−) c-Src and overexpressed wild-type EGFR, we show that the kinase activity of c-Src is required for both the biological synergy with the receptor and the phosphorylations on the receptor, but not for the association of c-Src with the receptor. In transient transfection assays, not only epidermal growth factor but also serum- and lysophosphatidic acid-induced DNA synthesis was ablated in a dominant-negative fashion by a Y845F mutant of the EGFR, indicating that c-Src-induced phosphorylation of Y845 is critical for the mitogenic response to both the EGFR and a G protein-coupled receptor (lysophosphatidic acid receptor). Unexpectedly, the Y845F mutant EGFR was found to retain its full kinase activity and its ability to activate the adapter protein SHC and extracellular signal-regulated kinase ERK2 in response to EGF, demonstrating that the mitogenic pathway involving phosphorylation of Y845 is independent of ERK2-activation. The application of these findings to the development of novel therapeutics for human cancers that overexpress c-Src and EGFR is discussed.**

Considerable evidence has accumulated in recent years to suggest that cellular Src (c-Src) and members of the epidermal growth factor (EGF) receptor (EGFR) family are critical elements in the etiology of multiple human cancers. Both kinases are found overexpressed in many of the same types of tumors, including glioblastomas and carcinomas of the colon, breast, and lung (1–4), raising the question of whether they functionally interact to promote the growth of these malignancies. In breast cancer, overexpression of EGFR family members is estimated to occur in 60% or more of the cases (5), and overexpression of the family member HER2/NEU, has been associated with a poor prognosis for the disease (6). Recent reports have also described overexpression of c-Src in a significant majority of patients with breast cancer, a frequency that approaches 100% (1). Studies to assess the oncogenic potential of each kinase have shown that the EGFR is tumorigenic when overexpressed in cultured fibroblasts and activated by ligand (7, 8), but overexpression of c-Src alone is insufficient for malignant transformation (9, 10).

A possible role for c-Src in tumorigenesis was revealed when it was demonstrated in C3H10T½ murine fibroblasts that co-overexpression of c-Src and the EGFR resulted in a synergistic increase in EGF-induced DNA synthesis, growth in soft agar, and tumorigenesis, as compared with cells overexpressing either the EGFR or c-Src alone (11). This cooperation correlated with the EGF-dependent formation of a physical complex between c-Src and the EGFR (11), the appearance of two unique sites of tyrosine phosphorylation (Y845 and Y1101) on the c-Src-associated EGFR, and increased phosphorylation of receptor substrates (11). These results suggest that one mechanism by which c-Src could augment the mitogenic/tumorigenic activity of the receptor is by associating with and hyperactivating the receptor by phosphorylation of novel tyrosine residues. Co-overexpression, co-association, and phosphorylation of Y845 and Y1101 have also been observed in human tumor cells (12–15), suggesting that synergism between c-Src and the EGFR may occur in a subset of human tumors as well as in murine fibroblasts.

To determine whether phosphorylation of Y845 or Y1101 is critical to the biological synergy between c-Src and the EGFR and to determine whether c-Src is responsible for mediating the phosphorylations, we analyzed a panel of murine fibroblasts that overexpressed either wild-type (wt) c-Src (K+ c-Src) or kinase-defective c-Src (K− c-Src) alone or together with the EGFR for growth properties and the presence of a stable complex containing the EGFR and c-Src. We found that K− c-Src inhibits EGF-dependent growth in soft agar and tumorigenesis in nude mice even though it is still capable of associating with the receptor. However, K− c-Src was unable to mediate the phosphorylation of Y845 on the receptor. As a direct test of the requirement of this phosphorylation for receptor function, we engineered a variant receptor harboring a Y845F mutation in the EGFR and observed that this mutated receptor ablated EGF, serum, and lysophosphatidic acid (LPA)-induced DNA synthesis without inhibiting receptor kinase activity or activation of the extracellular signal-regulated kinase ERK2. The data support a model wherein phosphorylation of Y845 on the EGFR by c-Src is required for EGF-induced mitogenesis and tumorigenesis in a manner that appears to be independent of ERK2.

MATERIALS AND METHODS

Cell Lines. The derivation, characterization, and maintenance of the clonal C3H10T½ murine fibroblast cell lines Neo (control), K+ (wt chicken c-Src overexpressors), K− (A430V kinase-deficient, chicken c-Src overexpressors), EGFR (wt human EGFR overexpressors), and EGFR/K+ (wt EGFR/wt c-Src double overexpressors) have been described previously (10, 11, 16). EGFR/K− (wt EGFR overexpressors/kinase-

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Abbreviations: c-Src, cellular Src; EGF, epidermal growth factor; EGFR, EGF receptor; K+, wild-type c-Src; K−, kinase-deficient c-Src; wt, wild- type; LPA, lysophosphatidic acid; BrdUrd, 5-bromodeoxyuridine; HA, hemagglutinin; MAPK, mitogen-activated protein kinase; GPCR, G protein-coupled receptor.
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deficient c-Src) cell lines were derived by infection of K− cells with a recombinant amphotropic retrovirus encoding the human EGFR (8), cloning by limiting dilution, and screening for overexpression of the receptor and maintenance of K− c-Src by Western immunoblotting. Clonal cell lines used in this study were estimated to express 25,000–60,000 human EGFRs per cell, based on comparative Western blotting analysis that used as a standard a C3H10T½ cell line that by Scatchard analysis was shown to express approximately 200,000 receptors per cell (5HR11 cells) (11). Clones included $EGFR_5$, $EGFR_8$, $EGFR_{27}$, $EGFR/K+_8$, $EGFR/K+_9$, $EGFR/K+_{10}$, and $EGFR/K-_2$, $EGFR/K-_5$, $EGFR/K-_{41}$, and $EGFR/K-_{56}$. K+ and K− c-Src overexpression was estimated to be 20- to 25-fold over endogenous.

Western Immunoblotting. Western blot analysis was performed as previously described (11, 16), using Ab-4 rabbit polyclonal antibody (Calbiochem) or F-4 mouse monoclonal antibody (mAb)(Sigma) to detect the EGFR, purified 2–17 mAb (Quality Biologicals; Gaithersburg, MD) or EC10 ascites fluid mAb (prepared in our laboratory and used at a 1:10,000 dilution) to identify c-Src, polyclonal anti-SHC antibody (Upstate Biotechnology; Lake Placid, NY) to visualize SHC, B3B9 mAb (17) to detect MAPK, polyclonal anti-phospho-MAPK antibody (Promega), or 4G10 anti-phosphotyrosine antibody (Upstate Biotechnology) to detect tyrosine-phosphorylated SHC. ^{125}I-labeled protein A (ICN) or ^{125}I-labeled goat anti-mouse or anti-rabbit Ig (New England Nuclear) and autoradiography were employed to localize binding of primary antibodies.

Colony Formation in Soft Agar and Tumorigenicity. Anchorage-independent growth was measured as previously described (11). Colonies were stained for 20 hr at 37°C in a solution of iodonitrotetrazolium violet (1 μg/ml; Sigma) in water and counted by using EAGLESIGHT analysis software (Stratagene). The soft agar colony data include analysis of three separate clones for each cell type, $EGFR_5$, $EGFR_8$, $EGFR_{27}$, $EGFR/K+_8$, $EGFR/K+_9$, $EGFR/K+_{10}$, $EGFR/K-_2$, $EGFR/K-_5$, and $EGFR/K-_{56}$. Assessment of tumor formation in Taconic *nu/nu* mice was performed as previously described (11).

***In Vitro* Kinase Assay, Metabolic Labeling with $^{32}P_i$, and Two-Dimensional Tryptic Phosphopeptide Analysis.** Methods for immunoprecipitation, *in vitro* kinase assay, metabolic $^{32}P_i$ labeling, and two-dimensional phosphopeptide analysis have been described (11, 15). In the metabolic labeling experiments, 5 μM pervanadate and 3 mM H_2O_2 were added to cells simultaneously with 100 ng/ml EGF and incubated for 5 min before harvesting.

5-Bromodeoxyuridine (BrdUrd) Incorporation. A pcDNA3 vector (Invitrogen) encoding human EGFR with a Y845F mutation was constructed by inserting a *Dra*III–*Bst*EII fragment containing the Y845F mutation (from plasmid pCO11, gift of L. Beguinot, Laboratory of Molecular Oncology, Milan, Italy) into the corresponding *Dra*III–*Bst*EII site of pcDNA3 encoding wt EGFR (gift of S. Decker, Parke–Davis, Ann Arbor, MI). K+ cells were transiently transfected with 4 μg of vector, wt EGFR, or Y845F EGFR plasmid DNA by using 30 μg of Superfect (Qiagen; Chatsworth, CA) according to manufacturer's directions and incubated in a humidified, 37°C, 5% CO_2/95% air atmosphere for 48 hr to allow a confluent monolayer to form. Transfected cells were then serum-starved for 30 hr prior to addition of 100 μM BrdUrd and 40 ng/ml EGF, 10% fetal bovine serum (FBS) in growth medium, or 10 μM LPA, at which time they were incubated for an additional 18 hr and costained for human EGFR expression and BrdUrd incorporation as described by the manufacturer of the BrdUrd-specific mAb (Boehringer Mannheim). Specifically, fixed cells were treated with 2 M HCl for 1 hr at 37°C and incubated with a mixture of primary antibodies (1:100 dilution of EGFR-specific Ab-4 and a 1:15 dilution of anti-BrdUrd mAb), followed by incubation with a mixture of secondary antibodies (75 μg/ml fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and 4 μg/ml Texas red-conjugated goat anti-mouse IgG, both from Jackson ImmunoResearch).

Transient Transfections. COS-7 cells were transiently transfected with plasmids encoding SHC and ERK2, by using Superfect as described above. HA-SHC (gift of K. Ravichandran; Univ. of Virginia; HA indicates influenza virus hemagglutinin) or Flag-ERK2 (gift of M. Weber; Univ. of Virginia) was transfected at a 1:5 ratio with or without either wt EGFR or Y845F mutant EGFR and incubated in a humidified, 37°C,

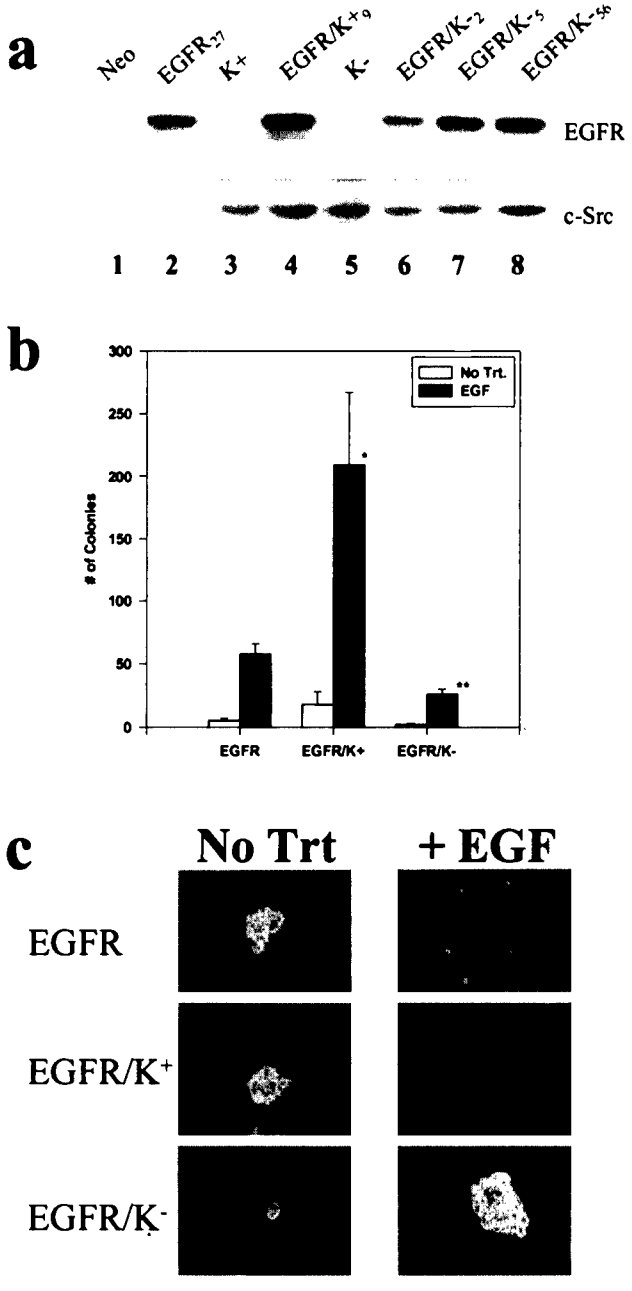

FIG. 1. Dominant repressive effect of K− c-Src on EGF-induced soft agar colony formation. (*a*) Western immunoblot analysis of C3H10T½ murine fibroblast clonal cell lines stably overexpressing EGFR and either wt (K+) or kinase-deficient (K−) c-Src. (*b*) Values for number of colonies are the mean ± SEM of at least six experiments in which 10^5 cells of each clone were seeded per plate in triplicate. Three clones of each cell type were averaged. *, $P < 0.04$ and **, $P < 0.002$ compared with EGFR. (*c*) Photomicrographs of representative fields of soft agar colonies formed from the indicated cell lines were taken after 2 weeks of growth. Trt, treatment. (×200.)

Table 1. K− c-Src completely ablates tumor formation in nude mice in 10T½ clones

Cell line	Tumor volume,* mm³
$EGFR_8$	295 ± 124
$EGFR/K+_9$	1,592 ± 598
EGFR/K−	0†

*Mean tumor volume ± SEM of eight individual sites was measured at day 52 after subcutaneous injection.
†EGFR/K− represents the mean tumor volume of two individual clones, $EGFR/K-_{41}$ and $EGFR/K-_{56}$.

5% CO_2/95% air atmosphere for 48 hr to allow a confluent monolayer to form. Transfected cells were then serum-starved overnight and either stimulated with 100 ng/ml EGF for 10 min or left untreated. Extracts were immunoprecipitated with either 12CA5 anti-HA antibody (Babco: Richmond, CA) or anti-Flag M2 affinity gel (Kodak) and resolved by SDS/PAGE.

RESULTS AND DISCUSSION

To determine whether phosphorylation of Y845 or Y1101 was dependent on c-Src catalytic activity, clonal C3H10T½ fibroblasts that stably overexpress wt EGFR and K− chicken c-Src were created from a stable K− c-Src expressing line, as described in *Materials and Methods*. Fig. 1*a* shows the levels of receptor and c-Src that are expressed in the various cell lines used in this study. EGFR/K− clones (lanes 6–8) expressed levels of receptor comparable to those in EGFR/K+ (lane 4) and EGFR (lane 2) lines, whereas all clones expressing K− c-Src (lanes 5–8) contained amounts of c-Src comparable to those of clones expressing K+ c-Src (lanes 3 and 4). Fig. 1*b* shows that the EGFR/K− clones exhibited diminished anchorage-independent growth in the presence of EGF compared with EGFR/K+ double overexpressors, demonstrating a requirement for the kinase activity of c-Src for potentiation of EGF-induced soft agar growth. Moreover, relative to cells overexpressing EGFR alone, the EGFR/K− clones also showed reduced soft agar growth, indicating that K− c-Src can function in a dominant-negative fashion for EGFR-induced colony formation. The dominant-negative effect was manifested by both reduced number (Fig. 1*b*) and significantly smaller average size (Fig. 1*c*) of the EGFR/K− colonies as compared with those of EGFR/K+ or EGFR cells. As previously reported, Neo control and K+ c-Src cells produced no or significantly fewer colonies than EGFR cells (11). K− c-Src cells also gave no colonies (data not shown). Table 1 shows that the growth of tumors *in vivo* was completely ablated in mice injected with EGFR/K− cells compared with EGFR or EGFR/K+ cells, demonstrating that K− c-Src has an even stronger dominant-negative effect on tumor growth *in vivo* than on growth in soft agar. Together these results underline the requirement for c-Src kinase activity in both the potentiating effect of overexpressed wt c-Src and the ability of overexpressed EGFR alone to induce oncogenic growth.

To determine whether K− c-Src might be eliciting its biological effects through the receptor, we examined the association between the two kinases, using an immune complex *in vitro* kinase assay as previously described (11). c-Src was immunoprecipitated from the C3H10T½ clones with a chicken c-Src-specific antibody, EC10, to minimize recognition of endogenous c-Src and to determine whether the exogenously expressed K− c-Src could interact with the EGFR. An EGF-sensitive *in vitro* phosphorylation of an ≈170-kDa protein was observed in the c-Src immunoprecipitates prepared from EGFR/K− (Fig. 2*a*, lanes 20, 23, and 26) as well as from EGFR/K+ cells (lanes 11 and 14). These results demonstrate that c-Src kinase activity is not required for association and suggest that K− c-Src may be eliciting its dominant negative effects (at least in part) directly through the receptor, since the association is still intact.

As described before (11), two tryptic phosphopeptides appear in the map of *in vitro* phosphorylated receptor associated with K+ c-Src (Fig. 3*B*) that are either absent or present in reduced amounts in the map of "free" activated receptor (Fig. 3*A*). These peptides contain Y845 and Y1101, whose identification is described in ref. 15. In contrast to the receptor associated with K+ c-Src, phosphorylation of Y845 was undetectable in receptor associated with K− c-Src (Fig. 3 *C* and *D*), while the level of Y1101 phosphorylation was visible but reduced. Similar results were observed in $^{32}P_i$ metabolic labeling experiments (Fig. 3 *E* and *F*). Phosphorylation on Y845 was also observed in cells expressing endogenous levels of c-Src, but only after treatment with pervanadate (15), suggesting that this site is phosphorylated in the absence of overexpression of c-Src and that it is rapidly turned over. These results indicate that phosphorylation of Y845, and to a lesser

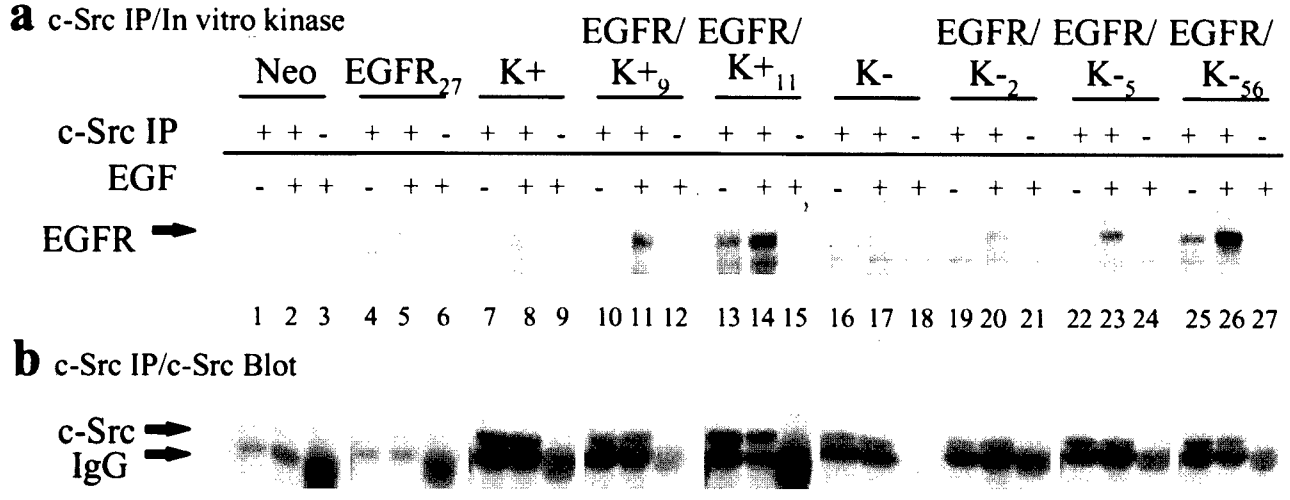

FIG. 2. K− c-Src associates with the EGFR. Confluent, serum-starved cells were untreated or treated with EGF (100 ng/ml) for 15 min, and extracts were immunoprecipitated with either chicken c-Src-specific mAb EC10 (designated +) or a negative control mouse IgG (−). (*a*) Immunocomplexes were subjected to an *in vitro* kinase assay using [γ-^{32}P]ATP, and phosphorylated products were analyzed by SDS/PAGE and autoradiography. The region of the autoradiograph around the 170-kDa products is shown. (*b*) The amount of c-Src in each precipitate was visualized by Western immunoblot analysis with EC10 mAb. K− c-Src in EGFR/K− clones was verified to be catalytically inactive in the immune complex kinase assay (data not shown).

extent of Y1101, depends on the kinase activity of c-Src, both *in vitro* and *in vivo*.

The position corresponding to Y845 is highly conserved among serine/threonine and tyrosine kinases and is situated in the activation loop between subdomains VII and VIII (18). Three-dimensional structural studies of several kinases have pointed to the importance of phosphorylation of this residue in stabilizing the activation loop in a conformation favorable for substrate and ATP binding (19–21). In agreement with the structural data, mutational analysis of the corresponding residue in tyrosine kinase receptors, including $p185^{neu}$, a highly conserved family member, has shown a requirement for phosphorylation of this residue for full biological function in response to ligand (22–27). Y845 homologues in other tyrosine kinase receptors have all been shown to be autophosphorylation sites. In contrast, Y845 of the EGFR has not been identified as such, and its importance to EGFR function has not been ascertained. The failure to identify Y845 as a site of autophosphorylation may reflect either the highly labile nature of the phosphorylation or the c-Src dependency of the phosphorylation (15, 28).

To determine whether phosphorylation of Y845 is required for receptor kinase activity, we compared wt EGFR autokinase activity with that of a mutant Y845F EGFR. Similar amounts of autophosphorylation were observed in an *in vitro* kinase assay of Y845F or wt EGFR immunoprecipitated from transiently transfected and EGF-stimulated COS-7 cells (Fig. 4). Further evidence that the EGFR, unphosphorylated on Y845F, retains its ability to autophosphorylate is provided by a comparison of the tryptic phosphopeptide maps of wt EGFR from EGFR cells (Fig. 3*A*) and wt EGFR from EGFR/K− cells

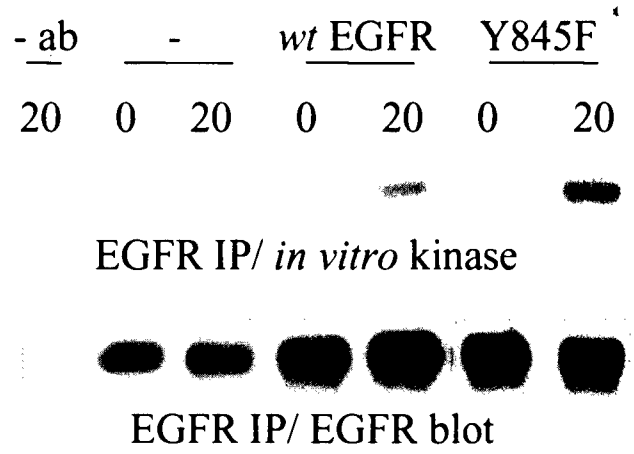

FIG. 4. Phosphorylation of Y845 is not essential for EGFR autokinase activity. COS-7 cells were transiently transfected with either vector alone (−), or plasmids encoding Y845F or wt EGFR and stimulated with EGF for 5 min. The EGFR was immunoprecipitated from extracts and subjected to an *in vitro* kinase assay for 0 or 20 min. The reaction was stopped with the addition of sample buffer, and the products were resolved by SDS/PAGE and transferred to a membrane. After autoradiography, EGFR was detected by Western immunoblotting and visualized by using enhanced chemiluminescence (ECL; Amersham).

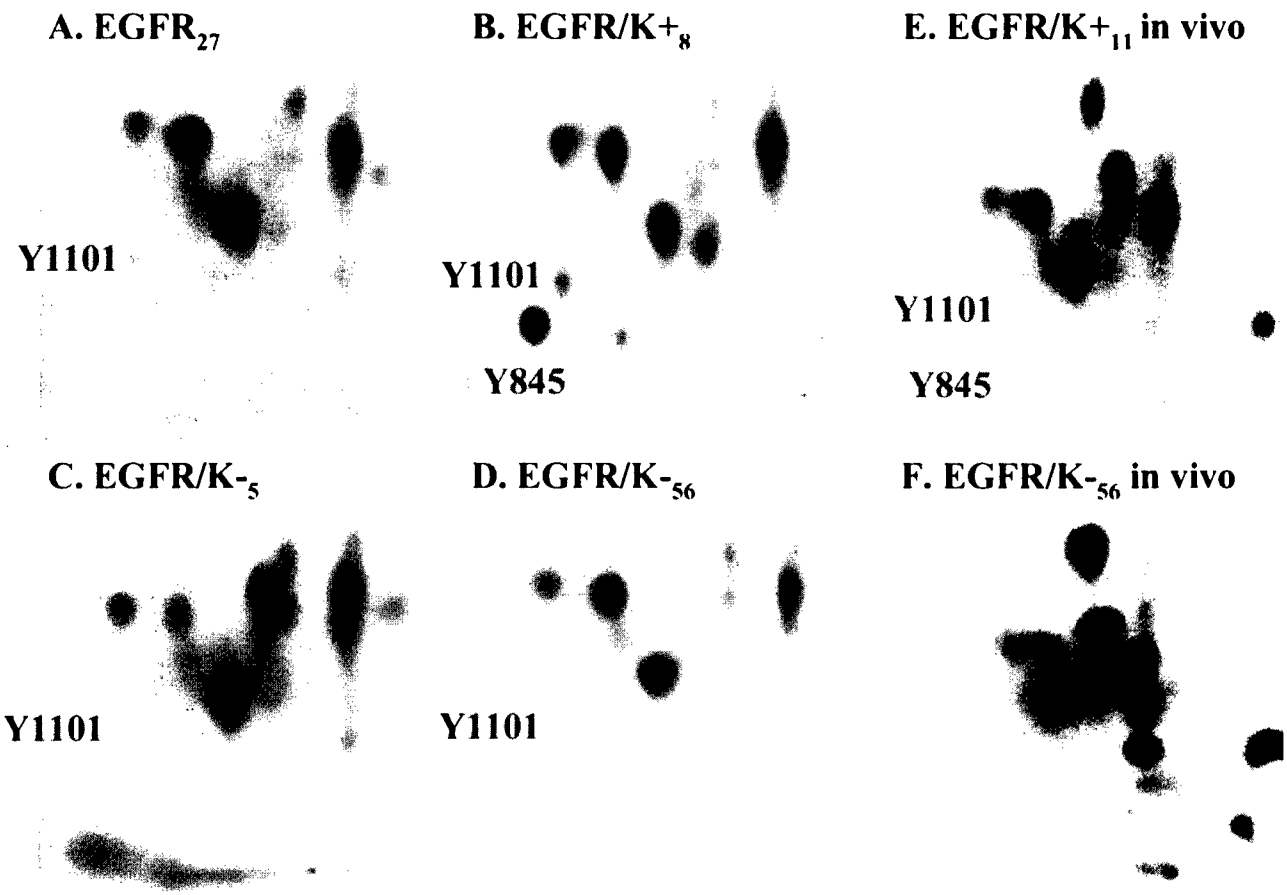

FIG. 3. Y845 is not phosphorylated in EGFR complexed with K− c-Src. (*A–D*) The 170-kDa bands that were phosphorylated *in vitro* (as in Fig. 2) in c-Src (*B–D*) or receptor (*A*) immunocomplexes prepared from the indicated cell lines were excised, digested with trypsin, resolved by two-dimensional electrophoresis/chromatography, and subjected to autoradiography. The positions of peptides containing Y845 and Y1101, which were identified previously (15), are indicated. (*E* and *F*) Receptor immunoprecipitates from the indicated cell lines that had been metabolically labeled with $^{32}P_i$ were analyzed as in *A–D*. Equal cpm were loaded in *A–D* and in *E* vs. *F*. The apparent increase in tyrosine phosphorylation in *F* is due to a slightly darker exposure compared with *E* to emphasize the complete ablation of Y845 phosphorylation. The appearance of darker or novel spots in *F* was not reproduced in repeated experiments.

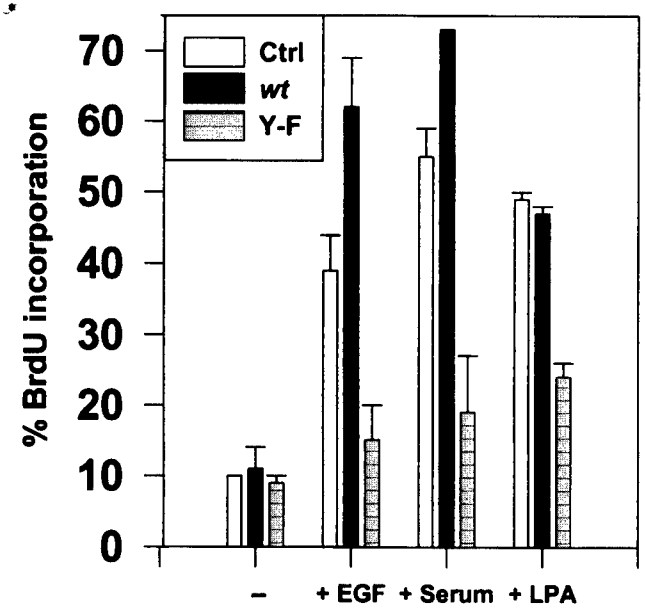

FIG. 5. Phosphorylation of Y845 is essential for EGFR function. K+ cells were transfected with plasmid DNA encoding Y845F (Y-F) or wt EGFR, cultured for 2 days, serum starved for 30 hr, and left untreated or treated with 40 ng/ml EGF, 10% fetal bovine serum, or 10 μM LPA for 18 hr. Results are expressed as the mean percent ± SEM of cells expressing EGFR that were positive for BrdUrd incorporation. Thirty-five to 100 cells were analyzed for each variable in three independent experiments.

(Fig. 3 *C* and *D*), where in the latter instance, autophosphorylation is maintained in the absence of detectable Y845 phosphorylation. These data suggest that, unlike other receptor tyrosine kinases mutated at the Y845 homologue, the Y845 mutant EGFR maintains its ability to autophosphorylate.

To test whether phosphorylation on Y845 is important for the mitogenic function of the EGFR independent of its autokinase activity, we transiently transfected a Y845F mutant or wt receptor into K+ cells and assessed mitogenesis by measuring EGF-induced BrdUrd incorporation into newly synthesized DNA. In contrast to the wt receptor, the Y845F mutant was unable to stimulate DNA synthesis upon EGF treatment (Fig. 5). Indeed, the reduced level of BrdUrd incorporation, which approached that of serum-starved cells, indicated that Y845F EGFR is capable of interfering with signaling through endogenous receptors, thereby acting in a dominant-negative fashion. These data support the hypothesis that phosphorylation of Y845 is required for the EGF-induced mitogenic function of the receptor.

Surprisingly, the Y845F variant of the EGFR also inhibited serum-induced DNA synthesis in a dominant-negative manner (Fig. 5). The mechanism of this inhibition is unclear at the present time. However, the EGFR has recently been shown to play an essential role in signaling and growth stimulation through G protein-coupled receptors (GPCR) (29), and the Src family of tyrosine kinases has also been directly implicated in GPCR-mediated mitogen-activated protein kinase (MAPK) activation (30, 31). c-Src is thought to be responsible for phosphorylating the EGFR in response to GPCR activation (32), leading to the generation of docking sites. The major mitogenic component of serum is LPA, a ligand for GPCR (33). Therefore, one possible mechanism by which the Y845F EGFR could prevent serum-induced BrdUrd incorporation might be the inability to phosphorylate Y845 via a GPCR route. Indeed, Y845F mutant EGFR was able to reduce (but not ablate) induction of DNA synthesis by LPA, demonstrating an involvement of EGFR signaling in the GPCR pathway (Fig. 5).

Because the EGFR is known to signal to MAPK via a SHC-Grb2-SOS-Ras pathway upon both EGF and G protein stimulation, we also tested the ability of the mutant EGFR to

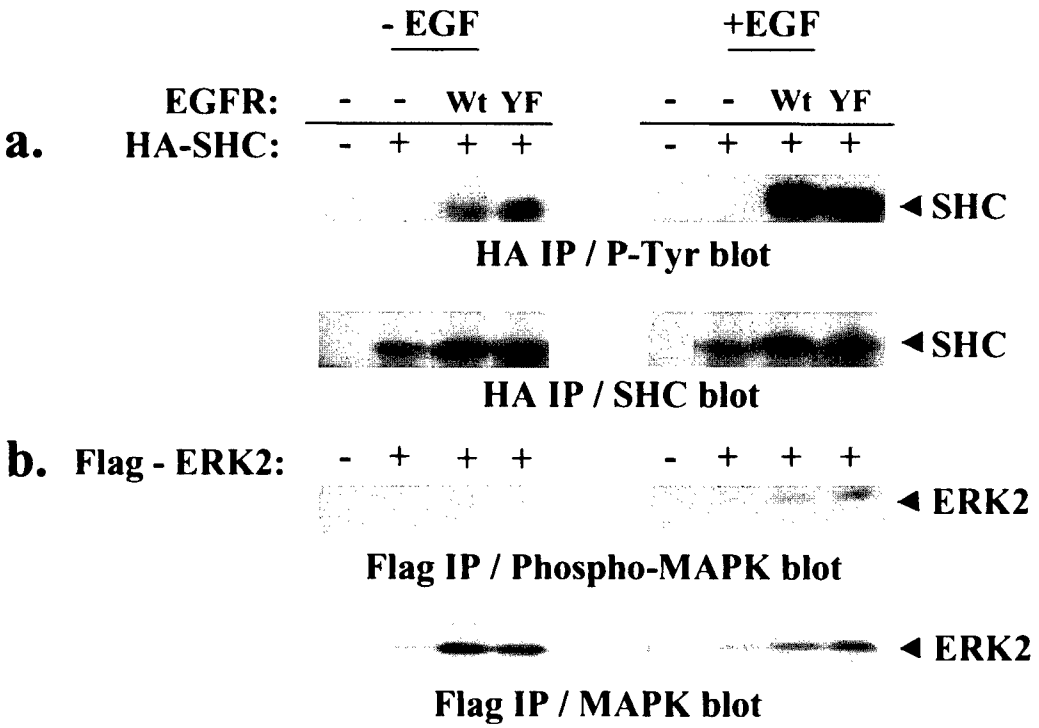

FIG. 6. Y845F mutant receptor retains its ability to phosphorylate SHC and activate MAPK. COS-7 cells were transfected with plasmid DNA encoding HA-SHC or Flag-ERK2 and either Y845F or wt EGFR, cultured for 2 days, serum starved overnight, and left untreated or treated with 100 ng/ml EGF for 10 min. Extracts were immunoprecipitated with 12CA5 anti-HA antibody or anti-Flag M2 affinity gel and resolved by SDS/PAGE. The amount of tyrosine phosphorylated HA-SHC (*a*) and Flag-ERK2 (*b*) was observed by Western immunoblot analysis. The amount of wt EGFR or Y845F EGFR expressed in the populations of cells was the same as that shown for Fig. 4.

phosphorylate the direct substrate SHC and to activate ERK2. The presence of the Y845F mutation in the EGFR did not alter the EGF-induced increase in tyrosine phosphorylation of cotransfected SHC or ERK2, compared with wt EGFR (Fig. 6). Minor differences in the relative phosphorylations of either SHC or ERK2 between mutant and wt EGFR were not significant when multiple experiments were quantitated (data not shown). These results suggest that the EGFR stimulates mitogenesis through an ERK2-independent pathway.

The data presented here provide a mechanism for c-Src's role in EGF- and GPCR-mediated DNA synthesis and tumorigenesis. We propose that phosphorylation of Y845 on the EGFR by a c-Src-mediated event is required for EGF- and LPA-induced DNA synthesis. On the basis of the findings that Y845 is not phosphorylated by the wt receptor alone (Fig. 3) and that the kinase activity of c-Src is required for phosphorylation of Y845, we conclude that c-Src is the most likely kinase to phosphorylate the receptor. Interruption of this phosphorylation by overexpressing a kinase-deficient c-Src or a Y845F mutant of the EGFR blocks signaling and thus growth. Furthermore, the block of DNA synthesis by Y845F mutant EGFR is not dependent on its ability to autophosphorylate or to signal to MAPK, suggesting a mechanism of activation in which the activation loop tyrosine does not need to be phosphorylated for kinase activity, but is required for stimulation of a mitogenic pathway not involving ERK2.

These findings have direct implications for the etiology of human cancers. In tumor cells that overexpress both c-Src and the EGF receptor, we postulate that the probability of Y845 phosphorylation increases, an event that results in promotion of growth and anchorage independence. Since phosphorylation of Y845 has been shown to occur in cultured human tumor cells that overexpress c-Src (15), the above paradigm may have relevance for the disease *in situ*. Development of methods to inhibit the ability of c-Src to phosphorylate Y845 may result in a novel, more "tumor-specific" treatment for cancers such as carcinomas of the colon, breast, and lung.

We thank Drs. S. Decker and L. Beguinot for wt and Y845F EGFR cDNA, respectively, D. Lowy and his lab for retroviral stocks, and members of the S.J.P. lab and the Parsons–Weber–Parsons group for helpful discussions. This work was supported by research grants CA71449 and CA39438 from the National Cancer Institute and 4621 from the Council for Tobacco Research (S.J.P.), a graduate fellowship (DAMD 17-96-6126) (D.A.T.), and a postdoctoral fellowship (DAMD 17-97-7329) (J.S.B.), both from the Department of Defense.

1. Ottenhoff-Kalff, A. E., Rijksen, G., van Beurden, E. A., Hennipman, A., Michels, A. A. & Staal, G. E. (1992) *Cancer Res.* **52,** 4773–4778.
2. Khazaie, K., Schirrmacher, V. & Lichtner, R. B. (1993) *Cancer Metastasis Rev.* **12,** 255–274.
3. Banker, N., Evers, B. M., Hellmich, M. R. & Townsend, C. J. (1996) *Surg. Oncol.* **5,** 201–210.
4. Biscardi, J. S., Tice, D. A. & Parsons, S. J. (1999) *Adv. Cancer Res.* **76,** in press.
5. Harris, J. R., Lippmann, M. E., Veronesi, U. & Willett, W. (1992) *N. Engl. J. Med.* **327,** 473–480.
6. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A. & McGuire, W. L. (1987) *Science* **235,** 177–182.
7. DiFiore, P. P., Pierce, J. H., Fleming, T. P., Hazan, R., Ullrich, A., King, C. R., Schlessinger, J. & Aaronson, S. A. (1987) *Cell* **51,** 1063–1070.
8. Velu, T. J., Beguinot, L., Vass, W. C., Willingham, M. C., Merlino, G. T., Pastan, I. & Lowy, D. R. (1987) *Science* **238,** 1408–1410.
9. Shalloway, D., Coussens, P. M. & Yaciuk, P. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7071–7075.
10. Luttrell, D. K., Luttrell, L. M. & Parsons, S. J. (1988) *Mol. Cell. Biol.* **8,** 497–501.
11. Maa, M.-C., Leu, T.-H., McCarley, D. J., Schatzman, R. C. & Parsons, S. J. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 6981–6985.
12. Luttrell, D. K., Lee, A., Lansing, T. J., Crosby, R. M., Jung, K. D., Willard, D., Luther, M., Rodriguez, J., Berman, J. & Gilmer, T. M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 83–87.
13. Stover, D. R., Becker, M., Liebetanz, J. & Lydon, N. B. (1995) *J. Biol. Chem.* **270,** 15591–15597.
14. Biscardi, J. S., Belsches, A. P. & Parsons, S. J. (1998) *Mol. Carcinog.* **21,** 261–272.
15. Biscardi, J. S., Maa, M.-C., Tice, D. A., Cox, M. E., Leu, T.-H. & Parsons, S. J. (1999) *J. Biol. Chem.* **274,** in press.
16. Wilson, L. K., Luttrell, D. K., Parsons, J. T. & Parsons, S. J. (1989) *Mol. Cell. Biol.* **9,** 1536–1544.
17. Reuter, C. W., Catling, A. D. & Weber, M. J. (1995) *Methods Enzymol.* **255,** 245–256.
18. Hanks, S. J., Quinn, A. M. & Hunter, T. (1988) *Science* **241,** 42–52.
19. Yamaguchi, H. & Hendrickson, W. A. (1996) *Nature (London)* **384,** 484–489.
20. Hubbard, S. R. (1997) *EMBO J.* **16,** 5572–5581.
21. Russo, A. A., Jeffrey, P. D. & Pavletich, N. P. (1996) *Nat. Struct. Biol.* **3,** 696–700.
22. Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A. & Rutter, W. J. (1986) *Cell* **45,** 721–732.
23. Fantl, W. J., Escobedo, J. A. & Williams, L. T. (1989) *Mol. Cell. Biol.* **9,** 4473–4478.
24. van der Geer, P. & Hunter, T. (1991) *Mol. Cell. Biol.* **11,** 4698–4709.
25. Vigna, E., Naldini, L., Tamagnone, L., Longati, P., Bardelli, A., Maina, F., Ponzetto, C. & Comoglio, P. M. (1994) *Cell. Mol. Biol.* **40,** 597–604.
26. Mohammadi, M., Dikic, I., Sorokin, A., Burgess, W. H., Jaye, M. & Schlessinger, J. (1996) *Mol. Cell. Biol.* **16,** 977–989.
27. Zhang, H.-T., O'Rourke, D. M., Zhao, H., Murali, R., Mikami, Y., Davis, J. G., Greene, M. I. & Qian, X. (1998) *Oncogene* **16,** 2835–2842.
28. Sato, K. I., Sato, A., Aoto, M. & Fukami, Y. (1995) *Biochem. Biophys. Res. Commun.* **215,** 1078–1087.
29. Daub, H., Weiss, F. U., Wallasch, C. & Ullrich, A. (1996) *Nature (London)* **379,** 557–560.
30. Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S. A. & Schlessinger, J. (1996) *Nature (London)* **383,** 547–550.
31. Luttrell, L. M., Hawes, B. E., van Biesen, T., Luttrell, D. K., Lansing, T. J. & Lefkowitz, R. J. (1996) *J. Biol. Chem.* **271,** 19443–19450.
32. Luttrell, L. M., Della Rocca, G. J., van Biesen, T., Luttrell, D. K. & Lefkowitz, R. J. (1997) *J. Biol. Chem.* **272,** 4637–4644.
33. Moolenaar, W., Kranenburg, O., Postma, F. R. & Zondag, C. M. (1997) *Curr. Opin. Cell Biol.* **9,** 168–173.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 12, Issue of March 19, pp. 8335–8343, 1999
Printed in U.S.A.
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c-Src-mediated Phosphorylation of the Epidermal Growth Factor Receptor on Tyr^{845} and Tyr^{1101} Is Associated with Modulation of Receptor Function*

(Received for publication, September 25, 1998, and in revised form, January 8, 1999)

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Accumulating evidence indicates that interactions between the epidermal growth factor receptor (EGFR) and the nonreceptor tyrosine kinase c-Src may contribute to an aggressive phenotype in multiple human tumors. Previous work from our laboratory demonstrated that murine fibroblasts which overexpress both these tyrosine kinases display synergistic increases in DNA synthesis, soft agar growth, and tumor formation in nude mice, and increased phosphorylation of the receptor substrates Shc and phospholipase γ as compared with single overexpressors. These parameters correlated with the ability of c-Src and EGFR to form an EGF-dependent heterocomplex *in vivo*. Here we provide evidence that association between c-Src and EGFR can occur directly, as shown by receptor overlay experiments, and that it results in the appearance of two novel tyrosine phosphorylations on the receptor that are seen both *in vitro* and *in vivo* following EGF stimulation. Edman degradation analyses and co-migration of synthetic peptides with EGFR-derived tryptic phosphopeptides identify these sites as Tyr^{845} and Tyr^{1101}. Tyr^{1101} lies within the carboxyl-terminal region of the EGFR among sites of receptor autophosphorylation, while Tyr^{845} resides in the catalytic domain, in a position analogous to Tyr^{416} of c-Src. Phosphorylation of Tyr^{416} and homologous residues in other tyrosine kinase receptors has been shown to be required for or to increase catalytic activity, suggesting that c-Src can influence EGFR activity by mediating phosphorylation of Tyr^{845}. Indeed, EGF-induced phosphorylation of Tyr^{845} was increased in MDA468 human breast cancer cells engineered to overexpress c-Src as compared with parental MDA 468 cells. Furthermore, transient expression of a Y845F variant EGFR in murine fibroblasts resulted in an ablation of EGF-induced DNA synthesis to nonstimulated levels. Together, these data support the hypothesis that c-Src-mediated phosphorylation of EGFR Tyr^{845} is involved in regulation of receptor function, as well as in tumor progression.

The epidermal growth factor receptor (EGFR)[1] is a 170-kDa single-pass transmembrane tyrosine kinase that undergoes homo- or heterodimerization and enzymatic activation following ligand binding (1, 2). These events result in the trans-(auto)-phosphorylation of multiple Tyr residues in the COOH-terminal tail of the molecule that serve as binding sites for cytosolic signaling proteins containing Src homology 2 (SH2) domains (3). Five sites of *in vivo* autophosphorylation have been identified in the EGFR: three major (Tyr^{1068}, Tyr^{1148}, and Tyr^{1173}) and two minor (Tyr^{992} and Tyr^{1086}) (4–7). These sites bind a variety of downstream signaling proteins which contain SH2 domains, including Shc (8) and PLCγ (9). Binding of these or other signaling proteins to the receptor and/or their phosphorylation results in transmission of subsequent signaling events that culminate in DNA synthesis and cell division.

c-Src is a nonreceptor tyrosine kinase that functions as a co-transducer of transmembrane signals emanating from a variety of polypeptide growth factor receptors, including the EGFR (see Refs. 10 and 11, and reviewed in Ref. 12). Overexpression of wild type (wt) and dominant negative forms of c-Src in murine C3H10T1/2 fibroblasts that express normal levels of receptor, as well as experiments involving the microinjection of antibodies specific for Src family members, have revealed that c-Src is a critical component of EGF-induced mitogenesis (10, 11, 13). Cells which express high levels of EGFR become transformed upon continual exposure to EGF (14), and co-overexpression of c-Src in these cells dramatically potentiates their growth and malignant properties (15). Together, these findings indicate that c-Src co-operates with the EGFR in the processes of both mitogenesis and transformation.

Subsequent studies in 10T1/2 cells revealed that potentiation of EGF-induced growth and tumorigenesis by c-Src, which is observed only in cells overexpressing both c-Src and the receptor, correlates with the EGF-dependent formation of a heterocomplex containing c-Src and activated EGFR, the appearance of two unique *in vitro* non-autophosphorylation sites on receptors in complex with c-Src, and enhanced *in vivo* tyrosyl phosphorylation of the receptor substrates, PLCγ and Shc (15). These findings suggested that c-Src-dependent phosphorylations on the EGFR can result in hyperactivation of receptor kinase activity, as measured by the enhanced ability of the receptor to phosphorylate its cognate substrates. This report identifies Tyr^{845} and Tyr^{1101} as c-Src-dependent sites of phosphorylation, which are present both *in vitro* and *in vivo* in receptor from 10T1/2 double overexpressing fibroblasts and

* This work was supported by United States Department of Health and Human Services Grants CA3948 and CA71449 (to S. J. P.), Council for Tobacco Research Grant 4621 (to S. J. P.), and Department of Defense Grants DAMD17-97-1-7329 (to J. S. B.) and DAMD17-96-1-6126 (to D. A. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: EGFR, epidermal growth factor receptor; DMEM, Dulbecco's modified Eagle's medium; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PLCγ, phospholipase Cγ; PIPES, 1,4-piperazinediethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; BrdUrd, bromodeoxyuridine; GST, glutathione *S*-transferase; mAb, monoclonal antibody.

from MDA468 human breast cancer cells. In the MDA468 cells, overexpression of c-Src results in a further increase in the phosphorylation of Tyr^{845}, indicating that c-Src either phosphorylates this site directly or activates a secondary kinase which is responsible. Moreover, cells which transiently express EGFR bearing a Tyr to Phe mutation at Tyr^{845} are impaired in their ability to synthesize DNA in response to EGF, suggesting that this c-Src mediated phosphorylation site is important for receptor function.

MATERIALS AND METHODS

Cell Lines—The derivation and characterization of the clonal C3H10T1/2 murine fibroblast cell lines used in this study, Neo (control), 5H (c-Src overexpressor), NeoR1 (human EGFR overexpressor), and 5HR11 (c-Src/EGFR double overexpressor) have been described previously (10, 11, 13). 5H and 5HR11 express equal levels of c-Src (~25-fold over endogenous), and NeoR and 5HR11 express nearly equal levels of cell surface receptors (~2×10^5 receptors/cell or ~40-fold over endogenous). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc., Gaithersburg, MD), containing 10% fetal calf serum, antibiotics, and G418 (400 μg/ml). When indicated, confluent cultures were starved of serum overnight, prior to stimulation with 100 ng/ml purified mouse EGF (Sigma).

To create cells transiently overexpressing HER1 which contained a Tyr to Phe mutation at position 845, a *Dra*III-*Bst*EII fragment from pCO11 (gift of Laura Beguinot), including the mutation at position 845, was subcloned into a pcDNA vector containing wild type human HER1 (gift of Dr. Stuart Decker, Parke Davis, Ann Arbor, MI). Neo control 10T1/2 fibroblasts were transiently transfected with 30 μg of Superfect (Qiagen, Chatsworth CA) and 4 μg of vector, wt HER1, or Y845F HER1 plasmid DNA according to the manufacturers' directions and incubated for 48 h.

For overexpression of c-Src in breast cancer cells, pcDNAc-Src was constructed by inserting the c-Src *Xho*I fragment from an existing pVZneo vector into the multicloning site of pcDNA3 (Invitrogen, San Diego, CA). MDA468 cells, obtained from N. Rosen (Sloan Kettering Cancer Center, New York), were maintained in DMEM plus 5% serum. MDA468 cells stably overexpressing chicken c-Src (clone MDA468c-Src) were generated by Lipofectin™ (Life Technologies, Inc.)-mediated gene transfer of pcDNAc-Src into parental MDA468 cells and selection with 400 μg/ml G418. Parental MDA468 cells overexpress c-Src approximately 5-fold, as compared with Hs578Bst normal breast epithelial cells, and contain approximately 10^6 receptors/cell (Ref. 16),[2] while MDA468c-Src cells overexpress c-Src approximately 25-fold over levels found in normal breast epithelial cells.

Antibodies—EGFR-specific mouse monoclonal antibodies (mAbs) 3A and 4A were provided by D. McCarley and R. Schatzman of Syntex Research, Palo Alto, CA. Their derivation has been described previously and their epitopes have been mapped to residues 889–944 and 1052–1134, respectively. EGFR-specific mAb F4, directed against amino acids 985–996, was obtained from Sigma. GD11 antibody is directed against the SH3 domain of c-Src and was characterized previously in our laboratory (17, 18). Q9 antibody was raised in rabbits against the COOH-terminal peptide of c-Src (residues 522–533) and exhibits a higher affinity for c-Src than for other Src family members (19, 20). Antiphosphotyrosine (Tyr(P)) antibody (4G10) was purchased from UBI (Lake Placid, NY). Negative control antibodies included pooled and purified normal rabbit or mouse immunoglobulin.

Immunoprecipitation, Western Immunoblotting, and in Vitro Kinase Assays—Methods for immunoprecipitation, Western immunoblotting, and *in vitro* kinase assays have been described previously (10, 11, 15). Cells were lysed either in CHAPS detergent buffer (10 mM CHAPS, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin, and 0.5% aprotinin), or in RIPA detergent buffer (0.25% sodium deoxycholate, 1% Nonidet P-40, 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 50 μg/ml leupeptin, and 0.5% aprotinin). Protein concentrations of detergent lysates were determined by the BCA protein assay (Pierce, Rockford, IL). 500 μg of cell lysate was used for immunoprecipitations, and 50 μg was used for Western blotting. For Western immunoblotting, binding of primary murine or rabbit antibodies to Immobilon membranes was detected with either ^{125}I-labeled goat anti-mouse IgG (NEN Life Science Products Inc.) or ^{125}I-protein A (NEN) used at 1 μCi/ml, specific activity 100 μCi/ml. For kinase assays, immunoprecipitates were prepared in and washed twice in CHAPS buffer, then washed twice with HBS buffer (150 mM NaCl, 20 mM HEPES, pH 7.4). Each kinase reaction was conducted in 20-μl volumes containing 20 mM PIPES, pH 7.5, 10 mM $MnCl_2$, and 10 μCi of [γ-^{32}P]ATP (6000 Ci/mmol, NEN) for 10 min at room temperature. Incubations were terminated by addition of sample buffer, and labeled products were resolved by SDS-PAGE and visualized by autoradiography.

In Vitro Binding and Far Western Analysis Using GSTc-SrcSH2—The construction and preparation of GST fusion proteins containing the SH2 domain of c-Src was described previously (21). To reconstitute binding between tyrosyl-phosphorylated EGFR and the SH2 domain of c-Src, 2 μg of immobilized GST-c-SrcSH2 fusion protein was incubated with 100 μg of 10T1/2 cell lysate protein prepared in RIPA buffer. After 3 h gentle mixing at 4 °C, beads were washed three times with RIPA buffer, resuspended in SDS sample buffer, and boiled. Eluted proteins were separated by SDS-PAGE, transferred to Immobilon, and immunoblotted with either the Tyr(P) or the 3A/4A or F4 (Sigma) monoclonal EGFR antibodies.

To assess direct binding of GST-c-SrcSH2 to the EGFR, receptor from 500 μg of cell lysate protein in RIPA buffer was immunoprecipitated with 3A/4A mAbs. The resulting EGFR immunoprecipitates were resolved by SDS-PAGE, transferred to Immobilon membranes, and incubated with 1 mg/ml purified GST-c-SrcSH2 fusion protein in blocking buffer at 4 °C overnight. The membrane was then probed with 1 μg/ml affinity purified, polyclonal rabbit anti-GST antiserum in blocking buffer,[3] and immunoglobulin binding was detected by ^{125}I-protein A.

Metabolic Labeling—NeoR1 or 5HR11 cells were grown to 50–75% confluency in 150-mm dishes, washed with phosphate-free DMEM, and incubated for 18 h in phosphate-free DMEM containing 0.1% dialyzed fetal bovine serum and 1 mCi/ml [^{32}P]orthophosphate (NEN Life Science Products Inc.) in a final volume of 10 ml. For pervanadate treatment, labeling medium was adjusted to a concentration of 3 mM H_2O_2 and 5 μM Na_3VO_4 just prior to EGF stimulation. Cells were stimulated in the presence of pervanadate by addition of 100 ng/ml EGF to the labeling medium for 5 min, washed twice with phosphate-free DMEM, and lysed in CHAPS detergent buffer. Extract from an entire plate (approximately 1–2 mg of protein) was immunoprecipitated with c-Src or EGFR-specific antibodies as described above.

Two-dimensional Tryptic Phosphopeptide Analysis—Immunoprecipitates of *in vitro* or *in vivo* ^{32}P-labeled EGFR were resolved by SDS-PAGE. The EGFR was localized by autoradiography, excised from the gel, and digested with trypsin as described by Boyle *et al.* (22). Phosphotryptic peptides were separated by electrophoresis at pH 1.9 in the first dimension and ascending chromatography in the second dimension on cellulose thin layer chromatography (TLC) plates. Chromatography buffer contained isobutyric acid, 1-butanol, pyridine, acetic acid, H_2O (125:3.8:9.6:5.8:55.8). Migration of synthetic phosphopeptides was detected by spraying the dried TLC plate with a hypochlorite solution consisting of sequential sprays with 10% commercial Clorox, 95% ethanol, 1% potassium iodide, and saturated *o*-tolidine in 1.5 M acetic acid, as described in Stewart and Young (23).

High Performance Liquid Chromatography (HPLC)—For HPLC analysis of peptides derived from the EGFR associated with c-Src, ^{32}P-labeled phosphotryptic peptides were prepared as above and suspended in 0.05% trifluoroacetic acid. Peptides were injected into a Perkin-Elmer Series 4 Liquid Chromatograph equipped with a Vydac C18 column (4.6 × 250 mm) and eluted with increasing concentrations of acetonitrile (0 to 100%) at a flow rate of 1 ml/min, as described by Wasilenko *et al.* (24). 500-μl fractions were collected, and Cerenkov counts of each fraction were determined. Fractions containing peptides "0" and "3" were identified by two-dimensional TLC analysis for their ability to co-migrate with the appropriate peptide in a mixture of total *in vitro* phosphorylated receptor peptides. Appropriate fractions were then lyophilized and subjected to Edman degradation.

Edman Degradation—HPLC fractions of ^{32}P-labeled EGFR phosphotryptic peptides or spots eluted from TLC plates were subjected to automated Edman degradation, as performed by the University of Virginia Biomolecular Research Facility. Briefly, phosphorylated peptides were coupled to a Sequelon aryl amine membrane (25), washed with 4 × 1 ml of 27% acetonitrile, 9% trifluoroacetic acid, and 2 × 1 ml of 50% methanol, and transferred to an applied Biosystems 470A sequenator using the cartridge inverted as suggested by Stokoe *et al.* (26). The cycle used for sequencing was based on that of Meyer *et al.* (27), but modified

[2] N. Rosen, personal communication.

[3] J. H. Chang and S. Parsons, unpublished data.

by direct collection of anilinothiazolinone amino acids in neet trifluoroacetic acid as described by Russo *et al.* (28). Radioactivity was measured by Cerenkov counting.

Identification of Peptides 0 and 3—Phosphorylated peptides (corresponding to residues GMN(Y-P)LEDR, candidate for peptide 3; or E(Y-P)HAEGGK, candidate for peptide 0) were synthesized by the University of Virginia Biomolecular Research Facility. Synthetic peptides were mixed with oxidized *in vitro* labeled phosphotryptic peptides from c-Src-associated EGFR, separated on cellulose TLC plates, and visualized by spraying with the hypochlorite solution as described above. One candidate for peptide 3 (GMNYLEDR) was synthesized as a phosphopeptide and tested for comigration as above. Another candidate for peptide 3 (DPHY^{1101}QDPHSTAVGNPEYLNTVQPTCVNSTF DSPAHWAQK), which was too large to chemically synthesize, was tested by further digestion of *in vitro* labeled peptide 3 with a proline-directed protease (Seikagaku, Rockville, MD), according to the method of Boyle *et al.* (22). In brief, the spot corresponding to peptide 3 was scraped off the TLC plate, eluted with pH 1.9 buffer, and digested with 5 units of proline-directed protease in 50 mM ammonium bicarbonate at pH 7.6 at 37 °C for 1 h. Peptides were separated by two-dimensional electrophoresis as described above.

BrdUrd Incorporation—Neo control cells, which had been transfected with cDNAs encoding wild type EGFR, Y845F EGFR, or vector alone were cultured for 48 h, then serum starved for an additional 30 h prior to the administration of 100 μM BrdUrd and either 100 ng/ml EGF or 10% fetal calf serum in fresh growth medium. Treated cells were incubated for 18 h and co-stained for HER1 expression and BrdUrd incorporation as described by the manufacturer of the BrdUrd-specific antibody (Boehringer Mannheim). Briefly, fixed cells were treated with 2 N HCl for 1 h at 37 °C and incubated with a mixture of primary antibodies (1:100 dilution of the HER1-specific Ab-4, and a 1:15 dilution of anti-BrdUrd mouse antibody in serum-free medium for 1 h at 37 °C), followed by incubation with a mixture of secondary antibodies (75 μg/ml fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and 4 μg/ml Texas Red-conjugated goat anti-mouse IgG) for 1 h at 37 °C. Both secondary reagents were obtained from Jackson Immunoresearch Laboratories, West Grove, PA.

RESULTS

Direct Binding of c-Src SH2 Domain to the EGFR—Previous work from our laboratory demonstrated a synergistic interaction between c-Src and the EGFR which led to increased cell growth and tumor development (10, 11, 15). This functional synergism was most striking when cells overexpressed both c-Src and the EGFR (5HR cells) and correlated with the ability of c-Src and the EGFR to form specific, EGF-dependent heterocomplexes *in vivo*. The formation of this c-Src·EGFR complex raises the question of whether binding between c-Src and the EGFR occurs directly, or is mediated by another protein present in the complex. To test whether association could be mediated by a Tyr(P)-SH2 interaction, lysates from unstimulated and stimulated Neo, 5H, NeoR, or 5HR cells were incubated with a GST-c-SrcSH2 bacterial fusion protein linked to agarose beads, and precipitated proteins were probed with Tyr(P) antibody. Fig. 1, *panel A*, *lanes 4* and *8*, show that a tyrosyl-phosphorylated protein of 170 kDa was precipitated by GST-c-SrcSH2 from extracts of cells overexpressing the EGFR after activation of the receptor with EGF. This 170-kDa protein co-migrated with the EGFR precipitated with receptor-specific mAbs 3A/4A (data not shown). Other proteins that bound c-SrcSH2 included p125^{FAK} (21), which was detected in all the cell lysates, a 75–80-kDa protein, cortactin, which was most prominent in 5H cells (30), and a 62-kDa protein, presumed to be related to the 62-kDa "DOK" protein associated with p120$^{Ras\text{-}GAP}$ (31–34). These results suggest that *in vivo*, multiple Tyr(P)-containing proteins in addition to the EGFR are capable of interacting with c-Src via its SH2 domain and contribute to the highly tumorigenic phenotype of the double overexpressing cells. Incubation of cell extracts with GST-beads alone resulted in no detectable binding of Tyr(P)-containing proteins (data not shown).

To confirm that the 170-kDa protein was indeed the EGFR,

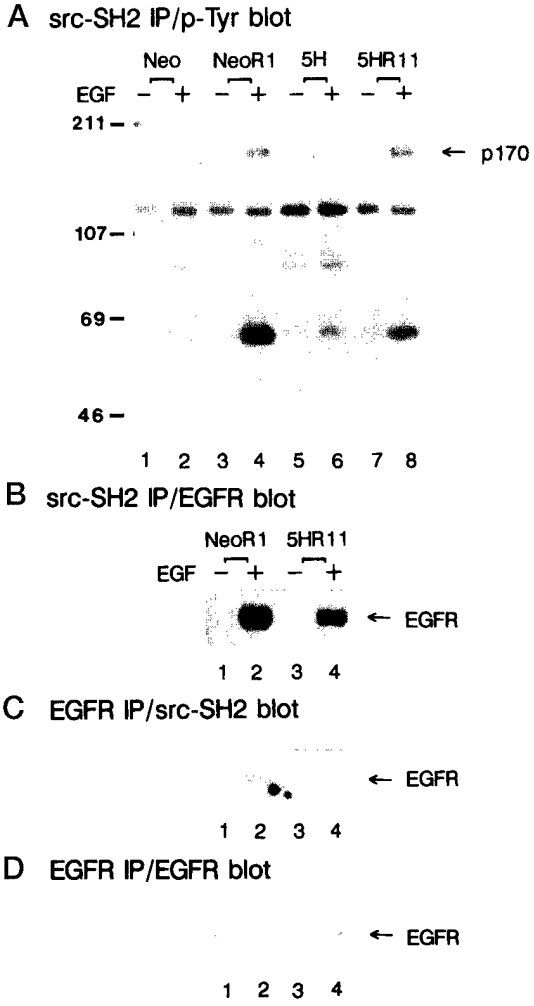

FIG. 1. ***In vitro* association between activated EGFR and c-Src SH2 domain.** 500 μg of lysate protein from the indicated nonstimulated cells or cells stimulated with 100 ng/ml EGF for 2 min was incubated with GST-c-SrcSH2 fusion protein immobilized on glutathione-agarose beads (*Panels A* and *B*) or EGFR mAbs 3A/4A bound to Protein A (*Panels C* and *D*), as described under "Materials and Methods." Affinity-precipitated proteins were washed and subjected to SDS-PAGE, transferred to Immobilon membranes, and probed with: *A*, Tyr(P) mAb 4G10; *B*, EGFR mAbs 3A/4A; *C*, GST-c-Src SH2 fusion protein; and *D*, EGFR mAb 3A/4A. Binding of primary antibody was visualized by incubating membranes with ^{125}I-labeled goat anti-mouse IgG (*Panels A*, *B*, and *D*), and binding of GST-c-SrcSH2 fusion protein was detected by rabbit anti-GST and ^{125}I-protein A (*Panel C*). GST-c-SrcSH2 fusion protein is shown to bind directly to activated EGFR.

lysates prepared from unstimulated and stimulated NeoR and 5HR cells were precipitated with immobilized GST-c-SrcSH2, and bound proteins were immunoblotted with EGFR-specific mAbs 3A/4A. Fig. 1, *Panel B*, demonstrates that receptor antibody detected the 170-kDa protein only in stimulated cells, as in *Panel A*, confirming its identity as the EGFR. To test if the interaction between the activated EGFR and c-SrcSH2 could be direct, receptor immunoprecipitates were subjected to a "Far Western" overlay experiment, using GST-c-SrcSH2, GST-specific antibody, and ^{125}I-protein A. Fig. 1, *Panel C*, *lanes 2* and *4*, shows that GST-c-SrcSH2 bound the EGFR and, as predicted, the interaction required activation by EGF. GST alone exhibited no binding (data not shown). *Panel D* verified that nearly equal amounts of receptor were present in all immunoprecipitates. These results provide evidence for the involvement of SH2-Tyr(P) interactions in the formation of the EGFR·c-Src complex.

In Vivo and in Vitro Phosphorylation of Novel, Non-auto-

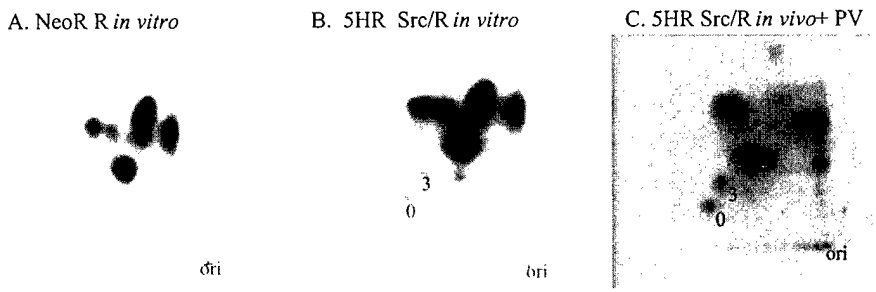

FIG. 2. **EGFR phosphotryptic peptides radiolabeled *in vitro* or *in vivo*.** For *in vitro* labeling (*Panels A* and *B*), 5HR and NeoR cells were stimulated with 100 ng/ml EGF for 30 min, followed by lysis in CHAPS buffer and immunoprecipitation of extract proteins with either c-Src-specific (GD11) or EGFR-specific (3A/4A) antibody. Precipitated proteins were then subjected to an *in vitro* kinase reaction, and products were analyzed by SDS-PAGE and autoradiography. For *in vivo* experiments (*Panel C*), cells were labeled for 18 h in phosphate-free media containing [^{32}P]orthophosphate, stimulated with 100 ng/ml EGF for 5 min in the presence of pervanadate, and lysed in CHAPS buffer. Extracts were immunoprecipitated with GD11 antibody, and precipitated proteins were analyzed by SDS-PAGE and autoradiography. c-Src-associated, ^{32}P-labeled EGFR was eluted from gel slices, and samples were trypsinized and analyzed by two-dimensional TLC as described previously (17). Labeled peptides were visualized by autoradiography. *Panel A, in vitro* labeled EGFR immunocomplexes from NeoR cells (2000 cpm); *Panel B, in vitro* labeled c-Src-associated EGFR from 5HR cells (2000 cpm); *Panel C*, c-Src-associated EGFR from 5HR cells labeled *in vivo* (3000 cpm). Tryptic maps were exposed to Pegasus Blue film (Pegasus, Burtonsville, MD) for 18 h.

phosphorylation Sites on the EGFR in Complex with c-Src—Overexpression of both EGFR and c-Src in 10T1/2 cells results in increased tyrosyl phosphorylation of receptor substrates, PLCγ and Shc, following EGF treatment (15). These findings suggest that the c-Src-associated receptor is modified in some manner as to increase its kinase activity. To examine the receptor for novel phosphorylations, the *in vitro* phosphorylated, c-Src-associated 170-kDa protein was excised from the gel and subjected to two-dimensional phosphotryptic peptide analysis. The phosphopeptide map of c-Src-associated receptor was then compared with the map of the free receptor, immunoprecipitated with receptor antibody. Fig. 2, *Panels A* and *B*, demonstrate that the maps are nearly identical; however, two additional phosphorylations (designated peptides 0 and 3) were seen in the map of the EGFR complexed with c-Src, suggesting that c-Src was responsible for their phosphorylation. Consistent with this notion, two-dimensional phosphoamino acid analysis of the *in vitro* labeled EGFR demonstrated that peptides 0 and 3 contained only phosphotyrosine (data not shown). *Panel C* shows that the two novel phosphopeptides were also detected in the receptor found in complex with c-Src from ^{32}P metabolically labeled 5HR cells that had been treated with pervanadate and EGF for 5 min. These data indicate that two phosphorylations occur on the EGFR both *in vitro* and *in vivo* when c-Src becomes physically associated with the receptor following EGF stimulation.

Initial attempts to detect peptides 0 and 3 in receptor immunoprecipitations from ^{32}P-labeled NeoR or 5HR cells yielded phosphopeptide maps that contained peptide 3 but no or barely detectable levels of peptide 0 (Fig. 3, *Panels A* and *C*). Neither could peptide 3 nor peptide 0 be detected reproducibly in receptor that was associated with c-Src from 5HR cells (data not shown). Furthermore, in receptor immunoprecipitations, the levels of peptide 3 derived from NeoR *versus* 5HR cells appeared nearly equal (compare *Panels A* and *C*), suggesting that peptide 3 may not be an *in vivo*, c-Src-dependent site of phosphorylation. In these experiments, lysates were prepared in CHAPS buffer containing a mixture of conventional protease and phosphatase inhibitors, including orthovanadate (see "Materials and Methods"). However, modification of the EGF treatment regimen to include pervanadate during stimulation allowed us to detect peptide 0 in receptor immunoprecipitates from NeoR (*Panel B*) and 5HR (*Panel D*) cells. These conditions revealed more peptide 0 in receptor from 5HR than from NeoR cells, confirming the ability of c-Src to modulate the phosphorylation of this peptide. Of special note was the finding that peptide 0 was the only peptide seen to increase in phosphorylation in response to pervanadate treatment, suggesting that its phosphorylation is more labile than that of peptide 3 or the other phosphorylations on the receptor, which presumably correspond to autophosphorylation sites. Together with the *in vitro* studies depicted in Fig. 2, the results from the *in vivo* experiments indicate that peptide 0 is an *in vitro* and *in vivo* site of receptor phosphorylation that is regulatable by c-Src. Following this line of reasoning, the low level of peptide 0 phosphorylation seen in receptor immunoprecipitates from NeoR cells (Fig. 3, *Panel B*) could be due to endogenous c-Src. However, the involvement of other tyrosine kinases in the *in vivo* phosphorylation of peptide 0 cannot be ruled out.

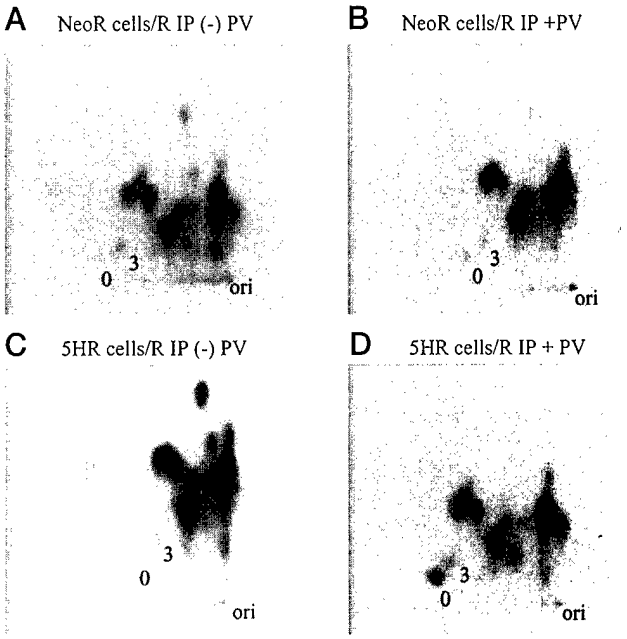

FIG. 3. **Phosphorylation of peptides 0 and 3 in metabolically labeled pervanadate-treated cells.** NeoR and 5HR cells were incubated for 18 h with [^{32}P]orthophosphate as above. Pervanadate (3 mM H_2O_2 and 5 μM Na_3VO_4) was added (*Panels B* and *D*) or not (*Panels A* and *C*) along with 100 ng/ml EGF for 5 min prior to lysis in RIPA detergent buffer. EGFR was immunoprecipitated with mAbs 3A/4A, and the receptor was processed for phosphotryptic analysis as described in the legend to Fig. 3. *Panel A*, EGFR from NeoR cells; *Panel B*, EGFR from pervanadate-treated NeoR cells; *Panel C*, EGFR from 5HR cells; *Panel D*, EGFR from pervanadate-treated 5HR cells. ~3000 cpm were loaded per TLC plate. TLC plates were exposed to Pegasus blue film for 18 h.

Whether c-Src alone plays a role in regulating the phospho-

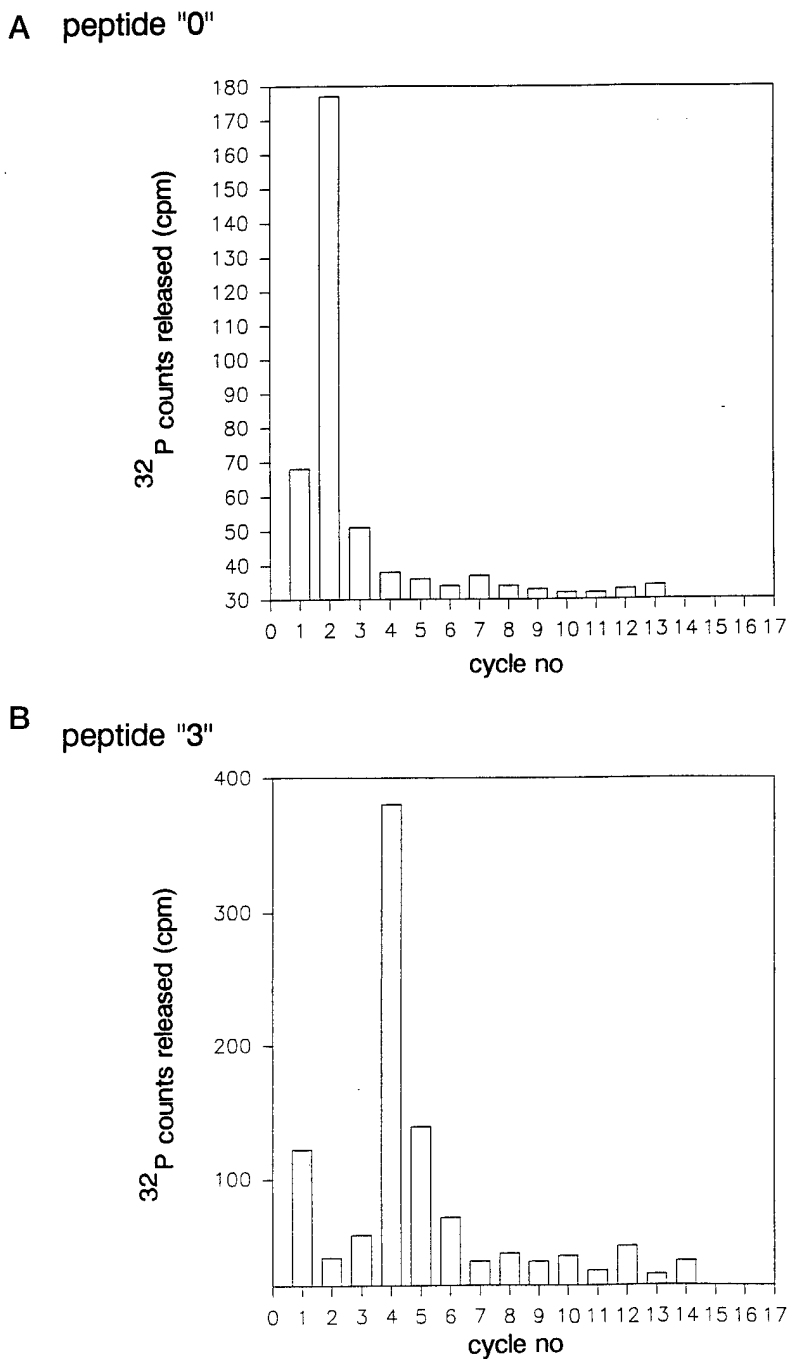

FIG. 4. **Edman degradation of peptides 0 and 3.** Peptides 0 and 3 were isolated by HPLC and subjected to automated Edman analysis. *A*, ^{32}P from peptide 0 was released at the second cycle, indicating a phosphorylated tyrosine at position 2; *B*, ^{32}P from peptide 3 was released at the fourth cycle, indicating a phosphorylated tyrosine at position 4.

rylation of peptide 3 *in vivo* is less clear. *In vitro*, peptide 3 phosphorylation appears to be unique to the receptor associated with c-Src (compare *Panels A* and *B* of Fig. 2), and HPLC analysis corroborates this, where phosphorylation of the peak corresponding to peptide 3 was found to be ~3.5-fold greater when the receptor was associated with c-Src *versus* free receptor (data not shown). Furthermore, the level of *in vivo* phosphorylation of peptide 3 in the c-Src-associated receptor is greater than that found in the "free" receptor (compare Fig. 2, *Panel C*, with Fig. 3, *Panel D*). However, peptide 3 is readily detected in free receptor labeled *in vivo*, and its level of phosphorylation does not appear to increase to any great extent in 5HR *versus* NeoR cells (Fig. 3, *Panels B* and *D*). These data can be interpreted to mean either that peptide 3 contains a nonlabile site of phosphorylation, regulatable by c-Src (in contrast to peptide 0), or that phosphorylation of peptide 3 may be regulated by an additional tyrosine kinase *in vivo*.

To identify the amino acids phosphorylated *in vitro* in a c-Src-dependent manner, fractions containing peptides 0 and 3 were isolated by HPLC. Peptide 0 eluted at 8.5% acetonitrile, while peptide 3 eluted at 10.5% acetonitrile (not shown). These HPLC fractions, which were of greater than 95% purity, were subjected to sequential Edman degradation to determine the cycle number at which radioactivity was released. Results from these analyses indicated that a phosphoamino acid residue was located at the second position of peptide 0 (Fig. 4, *Panel A*) and at the fourth position of peptide 3 (Fig. 4, *Panel B*). Of the tryptic peptides generated from the intracellular domain of the EGFR which contain Tyr residues, those peptides containing Tyr^{845}, Tyr^{867}, or Tyr^{891} were potential candidates for peptide 0, while those peptides containing Tyr^{803} or Tyr^{1101} were potential candidates for peptide 3 (see Table I).

TABLE I
Candidates for peptides 0 and 3

Peptide 0	**845** Glu-**Tyr**-His-Ala-Glu-Gly-Gly-Lys **867** Ile-**Tyr**-Thr-His-Gln-Ser-Asp-Val-Trp-Ser-Tyr-Gly-Val-Thr-Val Trp-Glu-Leu-Met-Thr-Phe-Gly-Ser-Lys **891** Pro-**Tyr**-Asp-Gly-Ile-Pro-Ala-Ser-Glu-Ile-Ser-Ser-Ile-Leu-Glu Lys
Peptide 3	**803** Gly-Met-Asn-**Tyr**-Leu-Glu-Asp-Arg **1101** Asp-Pro-His-**Tyr**-Gln-Asp-Pro-His-Ser-Thr-Ala-Val-Gly-Asn Pro-Glu-Tyr-Leu-Asn-Thr-Val-Gln-Pro-Thr-Cys-Val-Asn-Ser Thr-Phe-Asp-Ser-Pro-Ala-His-Trp-Ala-Gln-Lys

The Tyr^{845}-containing peptide was selected for further study as a candidate for peptide 0, since it showed 50% homology to sequences contained within the autophosphorylation site of Src (Tyr^{416}), indicating that it could be a potential c-Src target. The octamer composed of E(P-Y^{845})HAEGGK (peptide 0) was chemically synthesized to include a phosphorylated Tyr^{845} and analyzed either alone (Fig. 5, *Panel A*) or in a mixture with total peptides from *in vitro* labeled, c-Src-associated receptor by two-dimensional TLC (*Panel C*). The synthetic octamer comigrated with peptide 0 in the mixture, thereby identifying Tyr^{845} as the phosphorylated residue in peptide 0.

Since peptides 0 and 3 migrated similarly in the two-dimensional chromatography, it was expected that they would share similar isoelectric points and hydrophobicities. Both candidates for peptide 3 (GMNY^{803}LEDR or DPHY^{1101} QDPHSTAVGNPEYLNTVQPTCVNSTFDSPAHWAQK, see Table I) had theoretical isoelectric points and calculated hydrophobic indices (22) similar to those of the Tyr^{845}-containing peptide, indicating that both were potential candidates. The Tyr^{803}-containing peptide was selected first for further study, since it was smaller and more easily synthesized. However, this synthetic phosphopeptide did not co-migrate with peptide 3 nor with any of the other EGFR phosphopeptides (data not shown), indicating that the Tyr^{1101}-containing peptide was the preferred candidate. To verify the identity of peptide 3, *in vitro* labeled peptide 3 was scraped off the TLC plate, eluted with pH 1.9 buffer, and subjected to further digestion with a proline-directed protease as described under "Materials and Methods." Since, of the two candidate peptides, only the Tyr^{1101}-containing peptide contains proline residues, any change in mobility resulting from digestion with this protease would confirm its identity as peptide 3. As a control, peptide 0, which does not contain any proline residues, was digested with proline-directed protease and no change in mobility was observed (data not shown). Fig. 6 shows that digestion of spot 3 with the proline-directed protease resulted in a change of migration primarily in the first dimension (compare *Panel A* with *Panel B*). To confirm that a mobility shift was indeed occurring, digested and undigested peptide 3 were mixed (*Panel C*). The results identify peptide 3 as Tyr^{1101}.

Phosphorylation of Tyr^{845} and Tyr^{1101} in HER1 from Breast Tumor Cells—Our laboratory has previously demonstrated the presence of EGF- dependent c-Src·EGFR heterocomplexes in several human breast tumor cell lines including MDA468, which overexpresses both c-Src and HER1 (16). Since the presence of this heterocomplex is correlated with general increases in downstream receptor-mediated signaling and tumorigenicity in these cells, as compared with cell lines which do not overexpress the EGFR, we wished to investigate whether Tyr^{845} and/or Tyr^{1101} were phosphorylated in c-Src-associated EGFR derived from breast tumor cells. Fig. 7 demonstrates that phosphopeptides 0 and 3 are both present in *in vitro* labeled, c-Src-associated EGFR from EGF-stimulated MDA468 cells, although peptide 0 is weakly detected in the absence of pervanadate treatment. To further investigate the role of c-Src in mediating the phosphorylation of these sites, an MDA468 derivative cell line which stably overexpresses c-Src approximately 25-fold over levels in normal breast epithelial cells (MDA468c-Src cells, *Panel B*) was created. In these cells, the phosphorylation of peptide 0 (Tyr^{845}) was greatly enhanced, while the phosphorylation of peptide 3 (Tyr^{1101}) was unchanged (*Panel C*).

Role of Tyr^{845} in EGF-dependent Mitogenesis—A tyrosyl residue homologous to Tyr^{845} is conserved in many other receptor tyrosine kinases, and mutation of these conserved tyrosines to phenylalanine results in a reduced ability of the receptors to signal downstream events (35–37). Thus, it is possible that mutation of Tyr^{845} to phenylalanine would likewise decrease EGF-dependent signaling through the EGFR. To directly test the requirement of Tyr^{845} phosphorylation for receptor function, a variant receptor bearing a Y845F mutation was transiently transfected into Neo cells, and the effects on DNA synthesis were assayed by measuring bromodeoxyuridine (BrdUrd) incorporation in response to EGF (Fig. 8). The level of BrdUrd incorporation in cells expressing the Y845F mutant EGFR was reduced to approximately 30% of that induced by the wild type receptor, indicating that the mutant EGFR could interfere with the function of endogenous receptor and was thus acting in a dominant negative manner. Similar results were obtained when Y845F receptor was expressed in cells which overexpress c-Src (38). These findings suggest that phosphorylation of Tyr^{845} is necessary for the mitogenic function of the receptor.

DISCUSSION

Previous studies from our laboratory using the C3H10T1/2 murine fibroblast model demonstrated that simultaneous overexpression of c-Src and EGFR potentiates EGF-dependent mitogenesis, transformation, and tumorigenesis, as well as EGF-dependent association of c-Src with the receptor and increases in tyrosyl phosphorylation of the receptor substrates Shc and PLCγ (15). These events correlated with the appearance of two novel tyrosine phosphorylation sites on the receptor, suggesting that one mechanism by which c-Src could synergize with the EGFR is by physically complexing with it and mediating the phosphorylation of novel non-autophosphorylation tyrosine residues, which in turn may result in hyperactivation of the receptor and enhanced phosphorylation of receptor substrates. This increased signaling would then culminate in augmented cell division and tumor growth. Such a model was recapitulated in breast cancer cell lines of epithelial origin, wherein cell lines that express high levels of c-Src and EGFR exhibit EGF-de-

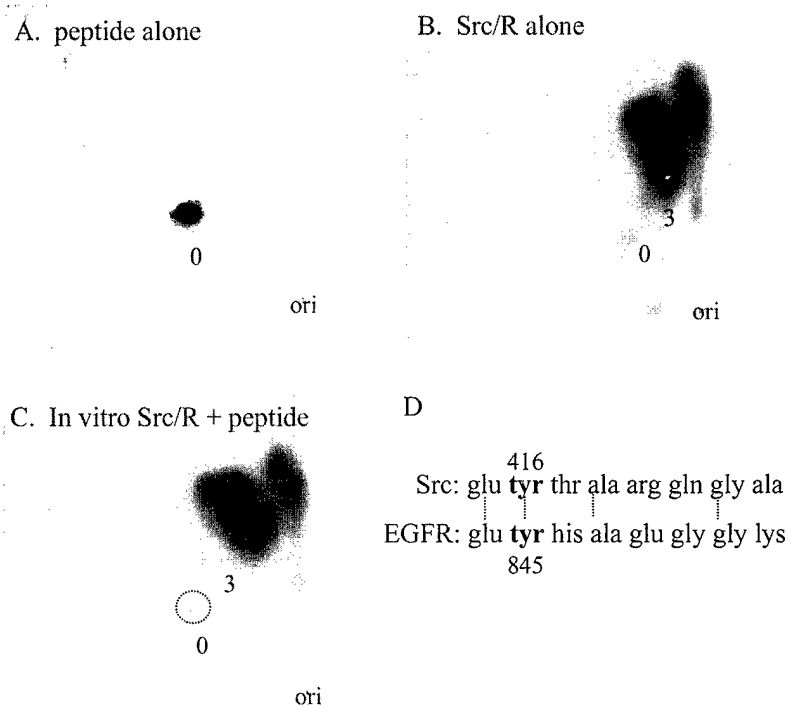

FIG. 5. **Identification of peptide 0.** The octapeptide E(Y-P)HAEGGK was synthesized to contain phosphorylated Tyr^{845} and analyzed by two-dimensional electrophoresis/chromatography on TLC plates, either alone (*Panel A*) or in a mixture with total *in vitro* labeled tryptic phosphopeptides derived from the receptor which co-precipitated with c-Src (*Panel C*). The synthetic phosphopeptide, detected by hypochlorite spraying, comigrated with tryptic peptide 0, verifying Tyr^{845} as the site on the receptor whose phosphorylation is dependent on c-Src. *Panel B*, total phosphopeptides from c-Src-associated receptor alone. *Panel D*, sequence homology between the peptide containing Tyr^{416} of c-Src and the peptide containing Tyr^{845} of the EGFR. 3000 cpm of *in vitro* labeled tryptic phosphopeptides were loaded along with 2 μg of synthetic phosphopeptide.

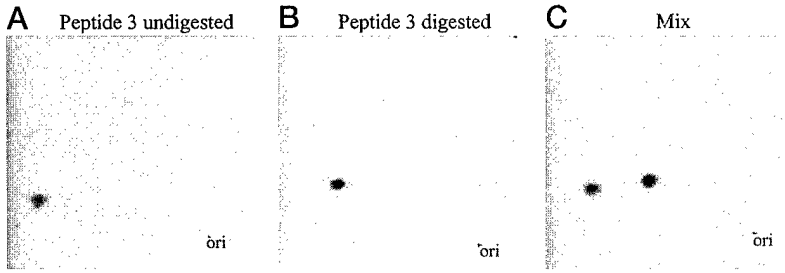

FIG. 6. **Identification of peptide 3.** *In vitro* phosphorylated peptide 3 (as in Fig. 5*B*) was scraped and eluted from the TLC plate and subjected to digestion with proline-directed protease. Undigested or digested, eluted peptide 3 was then analyzed by two-dimensional TLC either alone (*Panels A* and *B*, respectively) or mixed (*Panel C*). The altered mobility of digested peptide 3 indicates the presence of a proline in the sequence and identifies the peptide as containing Tyr^{1101}. 100 cpm of either digested or undigested peptide 3 were loaded on each TLC plate.

pendent association between c-Src and the receptor, augmented signaling through Shc and MAP kinase, and enhanced tumor formation, as compared with breast tumor cell lines which do not overexpress both c-Src and the EGFR (16). Because these and other studies link c-Src and the EGFR etiologically to tumorigenesis and malignant progression in many human tumors (reviewed in Ref. 12), identification of the two novel c-Src-dependent phosphorylations on the receptor and determination of their functions has taken on added importance, as they represent possible sites for therapeutic intervention.

Here we identify these c-Src dependent sites as Tyr^{845} and Tyr^{1101} and demonstrate that they become phosphorylated in murine fibroblasts both *in vitro* and *in vivo* in c-Src/EGFR double overexpressing cells in an EGF-dependent manner. Enhanced phosphorylation of Tyr^{845} was also observed in MDA468 human breast cancer cells when c-Src was overexpressed, indicating that such phosphorylations can occur in cells of both mesodermal and epithelial origin. More importantly, the fact that cells expressing a Y845F variant of the EGFR are impaired in their ability to synthesize DNA in response to EGF treatment provides direct evidence for the importance of this phosphorylation. Together, these findings support the hypothesis that the c-Src-mediated phosphorylation of Tyr^{845} is a critical event for EGFR function, and in certain situations where overexpression of these molecules exists (such as in certain breast tumors), the increased receptor signaling resulting from this phosphorylation could lead to enhanced tumorigenesis.

Tyr^{845} resides in an intriguing position on the receptor, namely in the activation lip of the kinase domain (39, 40). Amino acid sequences in this lip are highly conserved among tyrosine kinases (41). Crystallographic studies indicate that phosphorylation of Tyr^{845} homologues stabilizes the activation lip, maintains the enzyme in an active state, and provides a binding surface for substrate proteins; while mutation of these sites in their respective receptors results in decreases in cell growth and transformation (37, 40–43). A similar situation appears to exist for the EGFR, as cells expressing the Y845F variant receptor showed decreases in their ability to respond mitogenically to EGF. This impairment of DNA synthesis occurred both in a background of endogenous levels of c-Src, as shown here, as well as in cells where c-Src was overexpressed (38). This finding argues that endogenous levels of c-Src are capable of mediating the phosphorylation of Tyr^{845} and that the Y845F form of the receptor acts in a dominant negative fashion. Which downstream targets of the receptor are affected in various cell types by the Y845F mutation is not known. Other studies from our laboratory demonstrate that EGF-induced increases in Shc and mitogen-activated protein kinase tyrosyl

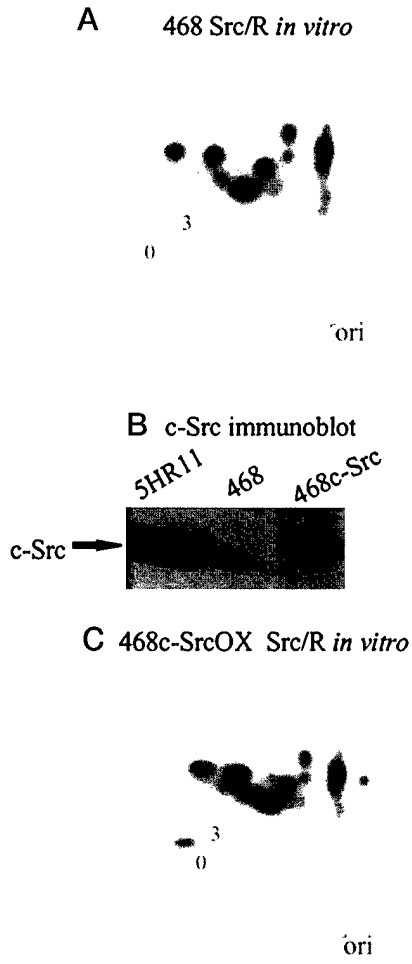

FIG. 7. **Phosphorylation of Tyr^{845} and Tyr^{1101} in MDA468 breast tumor cells.** MDA468 or MDA468c-Src cells were stimulated with 100 ng/ml EGF for 30 min, followed by lysis in CHAPS buffer and immunoprecipitation of extract proteins with either c-Src-specific (GD11) or EGFR-specific (F4) antibody. Precipitated proteins were then subjected to an *in vitro* kinase reaction. The labeled EGFR was eluted from gel slices, and samples were trypsinized and processed as described previously in the legend to Fig. 3. Labeled peptides were visualized by autoradiography. *Panel A,* phosphotryptic peptides from *in vitro* labeled EGFR immunocomplexes from MDA468 cells (4000 cpm). *Panel B,* protein extracts (50 μg) from MDA468 parental, 5HR, or MDA468c-Src cells which overexpress c-Src, were separated by SDS-PAGE and subjected to immunoblotting with GD11 antibody. *Panel C,* phosphotryptic peptides from *in vitro* labeled, c-Src-associated EGFR from MDA468c-Src cells (4000 cpm).

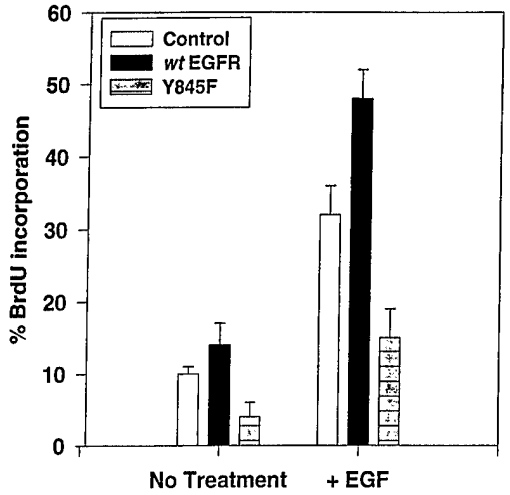

FIG. 8. **Phosphorylation of Tyr^{845} is required for EGF-induced DNA synthesis.** Neo control cells were transfected with plasmid DNA encoding Y845F or wild type EGFR, cultured for 2 days, serum starved for 30 h, and left untreated or treated with 40 ng/ml EGF for an additional 18 h. Cells were fixed and co-stained for EGFR expression and BrdUrd incorporation. Results are expressed as the mean percent ± S.E. of cells expressing EGFR that were positive for BrdUrd incorporation. Thirty-five to 75 cells were analyzed for each variable in three independent experiments.

phosphorylation occur normally when the Y845F receptor is transiently co-expressed in COS cells (38). This finding suggests that a mitogen-activated protein kinase-independent pathway plays a more dominant role in mitogenic signaling emanating from the receptor when it is phosphorylated on Tyr^{845}.

That phosphorylation of this Tyr^{845} residue may regulate receptor activity is consistent with the observation that a Tyr^{845} homologue is not found in the EGFR family member erbB3/HER3, which is known to lack kinase activity (44). However, unlike the situation resulting from mutation of the analogous site in other receptor tyrosine kinases, mutation of Tyr^{845} does not appear to alter the EGF receptor's ability to autophosphorylate or to phosphorylate the downstream substrate, Shc (38). In many tyrosine kinases, including Src, JAK 2, and receptors for colony stimulating factor-1, platelet-derived growth factor, insulin, and fibroblast growth factor, the Tyr^{845} homologue is an autophosphorylated residue (35, 36, 45–48). However, to date Tyr^{845} has not been identified as an autophosphorylation site for the EGF receptor. This could be due to the highly labile nature of the phosphorylation and/or to the fact that c-Src appears to regulate its phosphorylation (see Figs. 2, 3, and 7). Together these findings raise a number of questions: namely, whether c-Src phosphorylates Tyr^{845} directly, whether binding of c-Src to the receptor causes the receptor to phosphorylate itself, or whether another tyrosine kinase which mediates the phosphorylation is recruited into the complex or activated by c-Src.

Several pieces of evidence support the hypothesis that c-Src phosphorylates the receptor directly. First, Tyr^{845} is homologous to Tyr^{416} in Src, which is an autophosphorylation site for Src (39). Additional evidence comes from our studies with both 10T1/2 murine fibroblasts and MDA468 breast cancer cells overexpressing c-Src, where an enhanced phosphorylation of Tyr^{845} is observed. Moreover, other studies from our laboratory demonstrate that overexpression of a kinase inactive form of c-Src in 10T1/2 cells or in MDA468 cells results in a striking decrease in Tyr^{845} phosphorylation (38).[4] These latter findings indicate that c-Src kinase activity is necessary for the phosphorylation of Tyr^{845} and strongly argue that Tyr^{845} is a direct substrate of c-Src. Last, *in vitro* affinity precipitation and Far Western analyses (Fig. 1, this report, and Refs. 29, 49, and 50) demonstrate that the c-Src SH2 domain can bind activated EGFR specifically and directly, suggesting that recruitment of other tyrosine kinases is not necessary to mediate the phosphorylation of Tyr^{845}. However, other EGFR family members (including HER2/*neu*) (2, 51, 52) and several cytosolic tyrosine kinases, such as other c-Src family members (13) and JAK kinases (53, 54), have been reported to be involved in receptor-mediated signaling, and we cannot exclude their possible involvement in phosphorylation of Tyr^{845} or of Tyr^{1101}. Whether simple binding of c-Src induces a conformational change in the receptor so that it can autophosphorylate is a much more difficult question to address, a question that minimally awaits identification of the c-Src-binding site.

Other investigators have also described Src-mediated phosphorylations on the EGFR, and Wasilenko *et al.* (24) demon-

[4] J. S. Biscardi and D. A. Tice, unpublished results.

strated that in NIH3T3 cells co-expressing the transforming oncoprotein v-Src along with EGFR, the receptor contained several novel sites of tyrosine phosphorylation, one of which they postulated might be Tyr^{845} (SPY1). Sato *et al.* (55) provide additional evidence for phosphorylation of Tyr^{845} in A431 cells in a c-Src-dependent fashion, while Stover *et al.* (56) showed that Tyr^{891} and Tyr^{920} were phosphorylated in the c-Src-associated EGFR derived from MCF7 cells. However, neither we nor Sato *et al.* (55) have been able to detect phosphorylation of Tyr^{891} or Tyr^{920}, and none of these reports have linked the various phosphorylations to biological changes in receptor activity (*e.g.* mitogenesis, tumorigenesis). Thus, while there is some discrepancy among the different cell systems, our data and those of others indicate that Tyr^{845} is a major c-Src-dependent phosphorylation site on the EGFR, and that it is associated with increases in receptor function. These findings suggest that multiple tyrosine phopshorylations may be regulated by c-Src.

A potential role for Tyr^{1101} is more unclear, as this residue is not conserved among EGFR family members and its phosphorylation level *in vivo* is not as noticeably altered upon c-Src overexpression as is that of Tyr^{845} (see Fig. 3). However, Tyr^{1101} may function as a docking site for novel or known signaling proteins, perhaps in an SH2-dependent manner similar to that of the other autophosphorylation sites in the COOH terminus. One of the candidate binding proteins is c-Src itself. In peptide inhibition experiments using synthetic peptides to inhibit the binding between the EGFR and the SH2 domain of c-Src, the SH2 domain of c-Src was shown to bind Tyr^{992} (49, 55) and Tyr^{1101} (50) preferentially. Thus, c-Src could bind one of these sites, which could position it to phosphorylate Tyr^{845}. In MDA468 breast cancer cells, Tyr^{845} appeared to be the site most affected by c-Src. While the data from the 10T1/2 system suggests that the phosphorylation of both Tyr^{1101} and Tyr^{845} is dependent on c-Src, it may be that the phosphorylation of each peptide turns over at different rates in different cell types. Also, the endogenous levels of c-Src in the parental MDA468 cells may be capable of phosphorylating Tyr^{1101} to a maximal extent, and no further phosphorylation could result from overexpression. In this regard, overexpression of c-Src may allow for maximal phosphorylation of Tyr^{845} if this phosphorylation turns over at a faster rate, which appears to be the case as the results from Fig. 4 indicate.

Our data show that phosphorylation on Tyr^{845} appears to be critical for EGFR-mediated mitogenesis. Moreover, our results (Figs. 3 and 7) suggest that basal levels of c-Src are able to mediate phosphorylation of Tyr^{845} to some extent, and that this phosphorylation is important to receptor function. In a cell where overexpression and/or activation of c-Src has occurred, as is found in breast cancer, the proper negative regulation of this phosphorylation may be lost, resulting in the increased EGF-dependent signaling and tumorigenicity. We speculate that c-Src and EGFR act synergistically (via phosphorylation of the receptor by c-Src) to induce enhanced signaling in cells which overexpress both these kinases.

Acknowledgments—We thank Drs. John Shannon and Jay Fox of the Biomolecular Research Facility for Edman analysis, synthetic peptide production, and helpful advice in identification of peptide 3; Dr. Michael Weber for directing us toward comparisons of peptide 0 and "SPY1," and members of the Parsons-Weber-Parsons research group for critical discussion.

REFERENCES

1. Ullrich, A., and Schlessinger, J. (1990) *Cell* **61,** 203–212
2. Carraway, K. L., III, and Cantley, L. C. (1994) *Cell* **78,** 5–8
3. Pawson, T., and Schlessinger, J. (1993) *Curr. Biol.* **3,** 434–442
4. Downward, J., Parker, P., and Waterfield, M. D. (1984) *Nature* **311,** 483–485
5. Hsuan, J. J., Totty, N., and Waterfield, M. D. (1989) *Biochem. J.* **262,** 659–663
6. Margolis, B., Li, N., Koch, A., Mohammadi, M., Hurwitz, D. R., Zilberstein, A., Ullrich, A., Pawson, T., and Schlessinger, J. (1990) *EMBO J.* **9,** 4375–4380
7. Walton, G. M., Chen, W. S., Rosenfeld, M. G., and Gill, G. N. (1990) *J. Biol. Chem.* **265,** 1750–1754
8. Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Grignani, F., Pawson, T., and Pelicci, P. G. (1992) *Cell* **70,** 93–104
9. Rhee, S. G. (1991) *Trends Biochem. Sci.* **16,** 297–301
10. Luttrell, D. K., Luttrell, L. M., and Parsons, S. J. (1988) *Mol. Cell. Biol.* **8,** 497–501
11. Wilson, L. K., Luttrell, D. K., Parsons, J. T., and Parsons, S. J. (1989) *Mol. Cell. Biol.* **9,** 1536–1544
12. Biscardi, J. S., Tice, D. A., and Parsons, S. J. (1998) *Adv. Cancer Res.,* in press
13. Roche, S., Koegl, M., Barone, M. V., Roussel, M. F., and Courtneidge, S. A. (1995) *Mol. Cell. Biol.* **15,** 1102–1109
14. Velu, T. J., Beguinot, L., Vass, W. C., Willingham, M. C., Merlino, G. T., Pastan, I., and Lowy, D. R. (1987) *Science* **238,** 1408–1410
15. Maa, M-C., Leu, T-H., McCarley, D. J., Schatzman, R. C., and Parsons, S. J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 6981–6985
16. Biscardi, J. S., Belsches, A. P., and Parsons, S. J. (1998) *Mol. Carcinog.* **21,** 261–272
17. Parsons, S. J., McCarley, D., Ely, C. M., Benjamin, D. C., and Parsons, J. T. (1984) *J. Virol.* **51,** 701–705
18. Parsons, S. J., McCarley, D. J., Raymond, V. W., and Parsons, J. T. (1986) *J. Virol.* **59,** 755–758
19. Katagiri, T., Ting, J. P., Dy, R., Prokop, C., Cohen, P., and Earp, H. S. (1989) *Mol. Cell. Biol.* **9,** 4914–4922
20. Oddie, K. M., Litz, J. S., Balserak, J. C., Payne, D. M., Creutz, C. E., and Parsons, S. J. (1989) *J. Neurosci. Res.* **24,** 38–48
21. Cobb, B. S., Schaller, M. D., Leu, T. H., and Parsons, J. T. (1994) *Mol. Cell. Biol.* **14,** 147–155
22. Boyle, W. L., van der Geer, P., and Hunter, T. (1991) *Methods Enzymol.* **201,** 110–152
23. Stewart, J. M., and Young, J. D. (1984) *Solid Phase Peptide Synthesis*, Pierce Chemical Co., Rockford, IL
24. Wasilenko, W. J., Payne, M., Fitzgerald, D. L., and Weber, M. J. (1991) *Mol. Cell. Biol.* **11,** 309–321
25. Coull, J. M., Pappin, D. J. C., Mark, J., Aebersold, R., and Koster, H. (1991) *Anal. Biochem.* **194,** 110–120
26. Stokoe, D., Campbell, D. G., Nakielny, S., Hidaka, H., Leevers, S. J., Marshall, C., and Cohen, P. (1992) *EMBO J.* **11,** 3985–3994
27. Meyer, H. E., Hoffman-Posorske, E., Donella-Deana, A., and Korte, H. (1991) *Methods Enzymol.* **201,** 206–224
28. Russo, G. L., Vandenberg, M. T., Yu, I. J., Bae, Y.-S., Franza, B. R., Jr., and Marshak, D. R. (1992) *J. Biol. Chem.* **267,** 20317–20325
29. Luttrell, D. K., Lee, A., Lansing, T. J., Crosby, R. M., Jung, K. D., Willard, D., Luther, M., Rodriguez, M., Berman, J., and Gilmer, T. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 83–87
30. Maa, M.-C., Wilson, L. K., Moyers, J. S., Vines, R. R., Parsons, J. T., and Parsons, S. J. (1992) *Oncogene* **7,** 2429–2438
31. Ellis, C., Moran, M., McCormick, F., and Pawson, T. (1990) *Nature* **343,** 377–381
32. Chang, J.-H., Wilson, L. K., Moyers, J. S., Zhang, K., and Parsons, S. J. (1993) *Oncogene* **8,** 959–967
33. Carpino, N., Wisniewski, D., Strife, A., Marshak, D., Kobayashi, R., Stillman, B., and Clarkson, B. (1997) *Cell* **88,** 197–204
34. Yamanishi, Y., and Baltimore, D. (1997) *Cell* **88,** 205–211
35. Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A., and Rutter, W. J. (1986) *Cell* **45,** 721–732
36. Fantl, W. J., Escobedo, J. A., and Williams, L. T. (1989) *Mol. Cell. Biol.* **9,** 4473–4478
37. van der Geer, P., and Hunter, T. (1991) *Mol. Cell. Biol.* **11,** 4698–4709
38. Tice, D. A., Biscardi, J. S., Nickles, A. L., and Parsons, S. J. (1999) *Proc. Natl. Acad. Sci. U. S. A.*, in press
39. Cooper, J. A., and Howell, B. (1993) *Cell* **73,** 1051–1054
40. Hubbard, S. R., Wei, L., Ellis, L., and Hendrickson, W. A. (1994) *Nature* **372,** 746–754
41. Hanks, S. J., Quinn, A. M., and Hunter, T. (1988) *Science* **241,** 42–52
42. Longati, P., Bardelli, A., Ponzetto, C., Naldini, L., and Comoglio, P. M. (1994) *Oncogene* **9,** 49–57
43. Mohammadi, M., Dikic, I., Sorokin, A., Burgess, W. H., Jaye, M., and Schlessinger, J. (1996) *Mol. Cell. Biol.* **16,** 977–989
44. Guy, P. M., Platko, J. V., Cantley, L. C., Cerione, R. A., and Carraway, K. L. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 8132–8136
45. Piwnica-Worms, H., Saunders, K. B., Roberts, T. M., Smith, A. E., and Cheng, S. H. (1987) *Cell* **49,** 75–82
46. Kmiecik, T. E., and Shalloway, D. (1987) *Cell* **49,** 65–73
47. Roussel, M. F., Shurtleff, S. A., Downing, J. R., and Sherr, C. J. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 6738–6742
48. Feng, J., Witthuhn, B. A., Matsuda, T., Kohlhuber, F., Kerr, I. M., and Ihle, J. N. (1997) *Mol. Cell. Biol.* **17,** 2497–2501
49. Sierke, S. L., Longo, M., and Koland, J. G. (1993) *Biochem. Biophys. Res. Commun.* **191,** 45–54
50. Lombardo, C. R., Consler, T. G., and Kassel, D. B. (1995) *Biochemistry* **34,** 16456–16466
51. Bolen, J. B. (1993) *Oncogene* **8,** 2025–2031
52. Muthuswamy, S. K., and Muller, W. J. (1995) *Oncogene* **11,** 271–279
53. Leaman, D. W., Leung, S., Li, X., and Stark, G. T. (1996) *FASEB J.* **10,** 1578–1588
54. Schindler, C., and Darnell, J. E. (1995) *Annu. Rev. Biochem.* **64,** 621–651
55. Sato, K.-I., Sato, A., Aoto, M., and Fukami, Y. (1995) *Biochem. Biophy. Res. Commun.* **210,** 844–851
56. Stover, D. R., Becker, M., Leibetanz, J., and Lydon, N. B. (1995) *J. Biol. Chem.* **270,** 15591–15597

c-Src, Receptor Tyrosine Kinases, and Human Cancer

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I. INTRODUCTION

Since the discovery that tyrosine kinases are among the transforming proteins encoded by oncogenic animal retroviruses, it has been speculated that this family of enzymes may contribute to the development of human malignancies. However, evidence supporting that hypothesis has been slow to evolve, largely because early emphasis was placed on examining human tumors for genetic alterations in protooncogenes encoding these enzymes. Such alterations have proved rare or nonexistent. Instead, investigations have fo-

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Advances in CANCER RESEARCH
0065-230X/99 $30.00

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cused on determining levels of expression and posttranslational mechanisms of regulation of these proteins, particularly as they relate to signaling pathways that modulate growth, adhesion, invasion, and motility. Two classes of tyrosine kinases have emerged as potentially important players in promoting the evolution of human tumors: receptor kinases (RTKs) and nonreceptor tyrosine kinases of the c-Src family. Elevated levels of both these classes of tyrosine kinases can be found in a large number of tumors in a strikingly similar pattern of aberrant cooverexpression, suggesting that the two families may cooperate with one another during oncogenesis. Indeed, in model tissue culture systems, overexpression of receptor alone can result in malignant transformation when a continuous source of ligand is provided. However, overexpression of c-Src alone is non- or weakly oncogenic. These results indicate that c-Src, if it plays a role in tumorigenesis, most likely mediates its effects through RTKs. Demonstrations that c-Src physically associates with a number of RTKs in a ligand-dependent fashion provided some of the first evidence for functional cooperativity between these families of proteins. Subsequent studies showed that in complex, the two kinases reciprocally affect one another's behavior, such that c-Src can be regarded both as a regulator of RTKs and as a cotransducer of signals emanating from them. c-Src is capable of physically associating with the receptors for platelet-derived growth factor (PDGF), prolactin, epidermal growth factor (EGF), colony-stimulating factor-1 (CSF-1), fibroblast growth factor (FGF), and hepatocyte growth factor/scatter factor (HGF/SF), as well as with the HER2/*neu* and Sky tyrosine kinases (this review and Toshima *et al.*, 1995; Berlanga *et al.*, 1995), all of which are postulated to play a role in the genesis and/or progression of various human cancers. Although c-Src and its family members are also known to participate in signaling events elicited by heterotrimeric G protein-coupled receptors (Malarkey *et al.*, 1995) and neuronal ion channels (Ely *et al.*, 1994; Holmes *et al.*, 1996; Yu *et al.*, 1997; van Hoek *et al.*, 1997), this review focuses on the interactions of c-Src and Src family members with RTKs because of the growing documentation of the interactions between these proteins in human malignancies. First, a summary is presented, naming the RTKs that are most frequently implicated etiologically in human cancers and that have been shown to interact with c-Src. This summary includes a short review of the physical characteristics of the receptors, their molecular mechanisms of signaling, and their putative roles in specific cancers. Second, evidence is discussed for the involvement of c-Src and Src family members in human tumor development, and third, a synopsis is outlined showing the molecular mechanisms by which c-Src and its family members have been found to interact with receptors and other targets. Finally, we will speculate on the prospects for developing novel therapies based on these interactions.

II. RECEPTOR TYROSINE KINASES AND HUMAN CANCERS

Figure 1 depicts the structural features of several classes of RTKs that interact with c-Src. All consist of an extracellular ligand-binding domain that bears motifs characteristic of the type of receptor (e.g., repeated immunoglobulin-like motifs for the PDGF and FGF receptors or cysteine-rich motifs in the EGF family of receptors), a transmembrane segment, a tyrosine

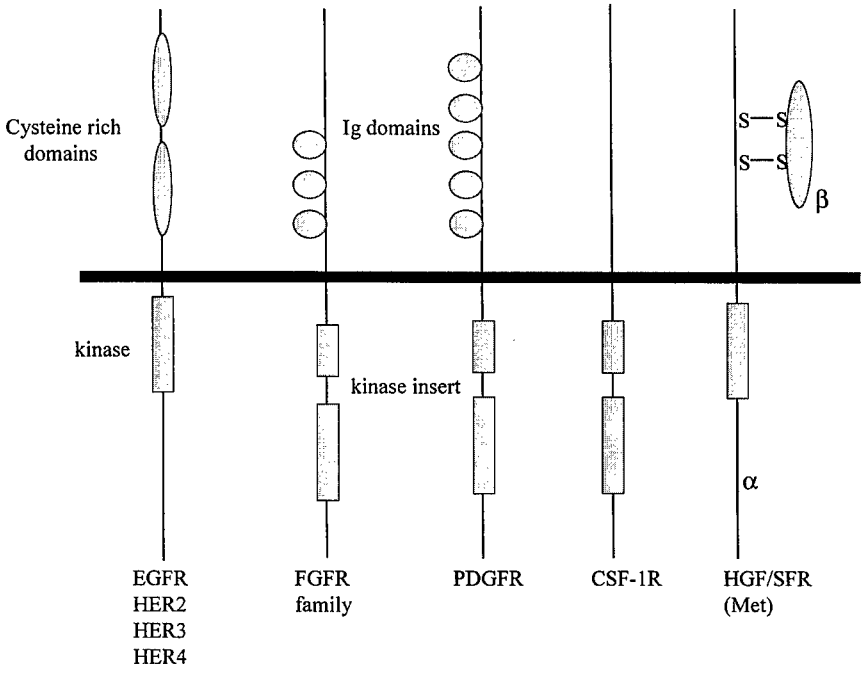


Fig. 1 Structures of receptor tyrosine kinase families known to associate with c-Src. All receptors are transmembrane glycoproteins that function as receptors for polypeptide growth factors. Structurally, these molecules are composed of large, extracellular domains that exhibit characteristic ligand-binding motifs, as well as transmembrane, juxtamembrane, catalytic, and C-terminal domains. Ligand binding induces dimerization, enzymatic activation, and autophosphorylation on specific tyrosine residues in the C-terminal domains. These phosphorylated tyrosine residues serve as docking sites for signaling molecules that transmit biological signals from the extracellular milieu to the nucleus. In the platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) receptor families, the kinase domain is interrupted by an insert that contains additional docking sites. EGFR, Epidermal growth factor receptor; CSF-1R, colony-stimulating factor-1 receptor; HGF/SFR, hepatocyte growth factor/scatter factor receptor; HER, human epidermal growth factor receptor.

kinase catalytic domain, and a carboxy-terminal region that contains sites of autophosphorylation. Binding of ligand causes dimerization of the receptor, activation of tyrosine kinase activity, and (trans) autophosphorylation of specific C-terminal tyrosine (Tyr) residues (reviewed in Heldin, 1996; Weiss *et al.*, 1997), which in turn serve as docking sites for a variety of signaling molecules that contain SH2 domains (Pawson and Schlessinger, 1993), including phospholipase Cγ (PLCγ), phosphatidylinositol-3 kinase (PI-3) kinase), GTPase-activating protein of Ras (RasGAP), phosphotyrosine phosphatases (PTPases), Janus kinases/signal transducers and activators of Transcription (JAK/STATS), adapter proteins (including Shc, Grb, Nck), and members of the c-Src family of tyrosine kinases (reviewed in Erpel and Courtneidge, 1995; Heldin, 1996). Signals are subsequently transmitted to the nucleus via several pathways, including the JAK/STAT and the Grb2/SOS/Ras/Raf/MEK/MAP kinase cascades (reviewed in Bonfini *et al.*, 1996; Denhardt, 1996). Members of the STAT and MAP kinase families translocate from the cytoplasm to the nucleus and induce changes in gene expression, which bring about a variety of functional outcomes, such as mitogenesis, morphogenesis, and motility. The contribution of c-Src to downstream signaling from these RTKs has been the subject of growing interest, with emphasis on how c-Src may contribute to transformation and maintenance of the cancerous phenotype that is dependent on and induced by the receptors.

In this treatise, a total of five RTK families and their putative roles in development of malignancy will be considered. The first four, receptors for HGF/SF, CSF-1, FGF, and PDGF, are implicated as etiological agents in a wide variety of human cancers, and their ability to influence processes such as cytoskeletal changes, cell motility, and angiogenesis are thought to contribute to the metastatic potential of tumors. The fifth group, members of the EGF receptor family (HER1–4), will be discussed in the context of breast cancer, along with the estrogen receptor. This steroid hormone receptor plays a pivotal role in the etiology of breast cancer and growing evidence indicates its ability to reciprocally interact with c-Src and members of the HER family of RTKs.

A. Hepatocyte Growth Factor/Scatter Factor Receptor

The Met tyrosine kinase is the receptor for hepatocyte growth factor/scatter factor (Bottaro *et al.*, 1991; Naldini *et al.*, 1991). This receptor was first identified as the product of the human oncogene, *tpr-met*, which was isolated from a chemically treated human cell line by the NIH3T3 gene transfer method (Cooper *et al.*, 1984; Park *et al.*, 1987). The normal cellular receptor is composed of two subunits, a 145-kDa β chain, which spans the cell

membrane and possesses ligand-binding and tyrosine kinase activity, and a 50-kDa α chain, which resides extracellularly and is covalently bound to the β subunit through disulfide linkages (Gonzatti-Haces *et al.*, 1988). Related family members include the Sea and Ron RTKs (Ronsin *et al.*, 1993; Huff *et al.*, 1993). Each member of the Met family possesses two tandemly arranged, degenerate YVH/NV motifs in the C-terminal tail of the receptor, which are capable of binding the SH2 domains of the signaling molecules PI-3 kinase, PTPase 2, PLCγ, c-Src, and Grb2/Sos (Ponzetto *et al.*, 1994). Mutations in these motifs (H1351N) result in increased transforming ability but decreased metastasis (Giordano *et al.*, 1997), a phenomenon that is linked to the creation of an additional Grb2 binding site and hyperactivation of the Ras pathway.

HGF/SF is produced by cells of mesodermal origin and acts on epithelial and endothelial cells, eliciting numerous biological responses, including cell motility, growth, morphogenesis, differentiation, and angiogenesis (Kan *et al.*, 1991; Rubin *et al.*, 1991; Halaban *et al.*, 1992). Which response is elicited in part depends on the cell type, developmental stage, and tissue context (Weidner *et al.*, 1993; Kanda *et al.*, 1993; Zhu *et al.*, 1994; Rosen and Goldberg, 1995; Grano *et al.*, 1996). For example, Met signals through STAT3 to induce the formation of branched tubule structures in Madin–Darby canine kidney (MDCK) cells, a hallmark of angiogenesis (Boccaccio *et al.*, 1998). HGF binding to primary human osteoclasts and osteoblasts triggers receptor kinase activity and autophosphorylation in both cell types. However, in osteoclasts, HGF binding is accompanied by increased levels of intracellular calcium, activation of c-Src, changes in cell shape, stimulation of chemotaxis, and DNA replication, whereas osteoblasts respond simply by undergoing DNA synthesis (Grano *et al.*, 1996). Furthermore, osteoclasts also express HGF, but osteoblasts do not. This finding suggests that an autocrine loop may be responsible for signaling in osteoclasts, whereas a paracrine mechanism is functional in osteoblasts.

HGF/SF and the Met receptor have been implicated in several types of human cancer. Met is overexpressed in gastric, ileal, colorectal, and thyroid papillary carcinomas, as well as in osteogenic sarcoma (Di Renzo *et al.*, 1991, 1992; Rosen *et al.*, 1994; Grano *et al.*, 1996). The level of Met expression, as measured by intensity of Met immunofluorescence, has also been shown to correlate with grade of malignancy in primary human brain tumors (Koochekpour *et al.*, 1997). In the case of ovarian carcinoma, Met levels can be regulated by the cytokines interleukin 1α (IL-1α), IL-6, and tumor necrosis factor α (TNFα), thereby providing a physiological mechanism by which overexpression of Met can be achieved (Moghul *et al.*, 1994). Approximately 14% of patients with papillary renal carcinoma have germ-line alterations in the Met receptor (Schmidt *et al.*, 1997). Receptors bearing these mutations have been shown in NIH3T3 cells to result in increased tyrosine kinase activity of the receptors and Met-mediated focus formation

and tumors in nude mice, thus providing direct evidence for the ability of mutationally altered Met to function as an oncogene (Jeffers *et al.*, 1997).

The ability of HGF/SF to "scatter" cells and to increase their motility is strongly suggestive of a role for this ligand in tumor cell invasion. Indeed, several lines of evidence link HGF/SF to stimulation of the urokinase plasminogen activator (UPA) system, a cascade of proteases thought to promote release, extravasation, and migration of tumor cells. That the UPA cascade is critical for cell migration is supported by the findings that UPA $-/-$ mice are unable to recruit migrating cells in response to inflammation (Gyetko *et al.*, 1996), and do not support the growth and metastasis of experimental melanomas (Min *et al.*, 1996). Shapiro *et al.* (1996) also showed that blocking interaction of UPA with its receptor results in decreased angiogenesis and tumor spread. The link between UPA and HGF was made when Jeffers *et al.* (1996b) reported that stimulation of the urokinase proteolytic system occurred concomitantly with HGF/SF-induced invasion and metastasis of human tumor cells. Rosen and Goldberg (1995) also demonstrated that HGF/SF is capable of stimulating angiogenesis in a rat cornea neovascularization assay. Together, these studies provide compelling evidence that HGF/SF are capable of promoting tumor progression by enhancing invasion and angiogenesis.

Further evidence for a role for HGF/S/Met receptor in tumor invasiveness and angiogenesis comes from the findings that high titers of HGF/SF in invasive breast cancers are factors for relapse and death (Yamashita *et al.*, 1994), that HGF/SF treatment of glioma cell lines stimulates proliferation and invasion (Koochekpour *et al.*, 1997), and that invasive bladder carcinomas possess higher HGF/SF titers than do noninvasive cancers (Joseph *et al.*, 1995). In addition, the Met receptor is overexpressed in several types of tumor stroma, including bladder wall, vascular smooth muscle, and vascular endothelial cells (Rosen and Goldberg, 1995), suggesting a paracrine signaling mechanism between tumor cells and the underlying stroma. Thus, HGF/SF and Met interactions may promote metastasis by enhancing proliferation via autocrine or paracrine routes, stimulating the expression of plasminogen activators, and triggering angiogenesis. (Rong *et al.*, 1992; Kanda *et al.*, 1993; Bellusci *et al.*, 1994; Jeffers *et al.*, 1996a,b).

B. Colony-Stimulating Factor-1 Receptor

c-Fms, the cellular homolog of the viral oncogene v-Fms (Sherr *et al.*, 1985), is the receptor for colony-stimulating factor-1, which stimulates the proliferation and differentiation of macrophages, osteoclasts, and placental trophoblasts (Sherr, 1990; Roth and Stanley, 1992; Insogna *et al.*, 1997). That CSF-1 is critical for the development of mononuclear phagocytes was

shown by studies in mice that fail to express functional CSF-1: these mice exhibit an osteopetrotic phenotype and lack osteoclasts and macrophages (Wiktor-Jedrzejczak *et al.,* 1990, 1991). The CSF-1 receptor is expressed in placenta (Pollard *et al.,* 1987; Regensstreif and Rossant, 1989; Hume *et al.,* 1997), osteolasts (Insogna *et al.,* 1997), and cells of monocyte lineage (Woolford *et al.,* 1985), whereas the ligand, CSF-1, is produced by fibroblasts, myoblasts, osteoblasts, bone marrow stromal cells, and endothelial cells (Sherr, 1990; Roth and Stanley, 1992). Such independent distribution of ligand and receptor underlies the importance of cell–cell interactions in regulating receptor function.

c-Fms bears sequence and structural similarity to the *steel* receptor, c-Kit, and to the receptors for FGF, PDGF, and Flt3/FLK2 (Hanks *et al.,* 1988; Rosnet and Birnbaum, 1993). The unique feature of this group is that each member possesses an "insert" region within its kinase domain. The downstream targets of c-Fms include PI-3 kinase, STAT 1, and PLCγ, all of which bind to phosphorylated tyrosine residues within the kinase insert portion of the molecule (Varticovski *et al.,* 1989; Shurtleff *et al.,* 1990; Reedijk *et al.,* 1990; Novak *et al.,* 1996; Bourette *et al.,* 1997). Bourette *et al.* (1997) have shown that sequential activation of the PI-3-kinase-dependent and PLCγ-dependent signaling pathways is required to initiate the differentiation process of myeloid cells. c-Src has also been shown to associate with c-Fms and to be activated on binding of CSF-1 to the receptor. Complex formation between c-Src and c-Fms is thought to occur via the SH2 domain of c-Src and a juxtamembrane phosphotyrosyl residue on the receptor (Courtneidge *et al.,* 1993; Alonso *et al.,* 1995). In osteoclasts, phosphorylation of c-Src in response to CSF-1 stimulation occurs concomitantly with rearrangements of the actin cytoskeleton and spreading of the cells, suggesting that c-Src may be involved in regulating these processes (Insogna *et al.,* 1997).

Overexpression of c-Fms in NIH3T3 or Rat2 fibroblasts or in various tumor cells results in transformation, growth in soft agar, and tumor formation in nude mice (Rettenmeier *et al.,* 1987; Taylor *et al.,* 1989; van der Geer and Hunter, 1989; Favot *et al.,* 1995). These findings demonstrate the oncogenic potential of overexpressed c-Fms. As described above, c-Fms and CSF-1 are normally not expressed in the same cell type. However, coexpression is seen in tumors of the pancreas, endometrium, stomach, lung, and breast, and in acute myeloid leukemia, hairy cell leukemia, and Hodgkin's lymphoma (Rambaldi *et al.,* 1988; Kacinski *et al.,* 1990; Paietta *et al.,* 1990; Baiocchi *et al.,* 1991; Kauma *et al.,* 1991; Bruckner *et al.,* 1992; Filderman *et al.,* 1992; Storga *et al.,* 1992; Tang *et al.,* 1992; Leiserowitz *et al.,* 1993; Till *et al.,* 1993; Burthem *et al.,* 1994; Berchuck and Boyd, 1995). Coexpression correlates with poor patient prognosis, most likely due to the establishment of an autocrine loop (Kacinski *et al.,* 1990; Tang *et al.,* 1992). Evidence suggests that such an autocrine loop contributes not only to tumor

cell proliferation but also to invasiveness (Bruckner *et al.*, 1992; Filderman *et al.*, 1992; Burthem *et al.*, 1994). In this regard, coexpression of c-Fms and its ligand in endometrial cancers correlates with a more advanced stage and with increased myometrial invasion (Leiserowitz *et al.*, 1993). Moreover, CSF-1 stimulation results in the expression of UPA in lung tumors, Lewis lung carcinoma cells, and in NIH3T3 cells transfected with c-Fms (Filderman *et al.*, 1992; Favot *et al.*, 1995; Stacey *et al.*, 1995). Together, these findings suggest that, like HGF/SF/MetR, deregulation of c-Fms/CSF-1 interactions has the potential of contributing to the metastatic process in a variety of human cancers.

C. Fibroblast Growth Factor Receptors

The FGF receptors comprise a large family that is encoded by four separate genes, each of which can be alternatively spliced. Each receptor is also capable of binding several different ligands, resulting in a complex array of possible receptor/ligand pairs (Johnson and Williams, 1993). All receptors for FGF possess extracellular ligand-binding domains, which contain immunoglobulin-like repeats, and bipartite, intracellular tyrosine kinase domains (Lappi, 1995). Which signaling molecules are recruited varies with cell type and receptor/ligand pair. For example, in NIH3T3 cells (Zhan *et al.*, 1994) FGFR 1 and c-Src physically associate following ligand binding, and activation of the receptor triggers the c-Src-dependent phosphorylation of the actin-binding protein, cortactin. Because cortactin is localized to cortical actin, particularly at the leading edge of a migrating cell (Wu *et al.*, 1991; Maa *et al.*, 1992; Wu and Parsons, 1993), its phosphorylation is speculated to influence cell motility and invasiveness. In other studies, ligand stimulation of FGFR 1 and FGFR 3 on C6 rat myoblasts results in activation of the p21Ras and MAPK pathway (Klint *et al.*, 1995; Kanai *et al.*, 1997). In these same cells, activation of the FGFR 3 receptor alone causes an increase in phosphorylation of PLCγ but a decrease in c-Src phosphorylation (Kanai *et al.*, 1997).

FGF receptors are ubiquitously expressed during embryogenesis, but their presence is restricted after birth (Wanaka *et al.*, 1991; Peters *et al.*, 1992, 1993; Pastone *et al.*, 1993). As a family, FGFs have mitogenic, nonproliferative, and antiproliferative effects. Which response is elicited is determined by the ligand, the type of cell exposed to the ligand, and the particular isoform of the receptor expressed on that cell (Schweigerer *et al.*, 1987; Sporn and Roberts, 1988). For example, FGF 2 promotes survival of cultured neurons (Walicke, 1988), whereas FGF 1 and FGF 2 stimulate growth of fibroblasts, oligodendrocytes, astrocytes, smooth muscle cells, endothelial

cells, and retinal epithelial cells (Burgess and Maciag, 1989). FGFs can also act as chemotactic factors for fibroblasts and glial cells (Senior *et al.,* 1986). Basic FGF (bFGF, or FGF 2) induces neurite outgrowth in embryonic chick ciliary ganglion cells (Schubert *et al.,* 1987) and can mediate cellular migration in experimental systems (Sato and Rifkin, 1988). Treatment of cultured vascular endothelial cells with FGF 2 induces the formation of blood capillary-like tubules, a finding that suggests FGFs may play a role in angiogenesis (Montesano *et al.,* 1986; Slavin, 1995). In this regard, a large literature is beginning to accumulate in support of a role for FGFs in angiogenesis, because they have been demonstrated to stimulate endothelial cell division, migration, release of proteolytic enzymes, and capillary formation (Slavin, 1995).

In addition to these functions in normal cells, FGFR family members are implicated in the progression of a variety of human cancers. FGFs are thought to act as autocrine growth factors for melanomas, gliomas, and meningiomas (Lappi, 1995), and their levels are elevated in many different tumor types (Nguyen *et al.,* 1994). FGF receptors are also overexpressed in human tumors. For example, 10% of human breast tumors exhibit amplifications of chromosomal regions encoding FGF receptors (Adnane *et al.,* 1991), and FGFR 4 mRNA levels are frequently elevated in breast cancer cells as compared to normal tissue (Lehtola *et al.,* 1993; Ron *et al.,* 1993; Penault-Llorca *et al.,* 1995). Some evidence also suggests that differential expression of FGFR isoforms can influence the propensity of a cell to undergo malignant transformation. In normal fetal and mature brain, FGFR 1, which possesses three immunoglobulin-like extracellular repeats, is expressed. However, in astrocytic tumors, an increase in the expression of an FGFR with two immunoglobulin-like domains is observed. This form has increased affinity for acidic and basic FGF (Shing *et al.,* 1993). Changes in FGFR expression also occur during the conversion of normal or hyperplastic prostatic epithelium to malignant tumor tissue, where the increased expression of an alternatively spliced form of FGFR 2, which has a higher affinity for bFGF, appears to create an autocrine stimulatory loop (Wang *et al.,* 1995).

FGFs, along with other factors, are often secreted by tumors, and their increased extracellular abundance is linked to enhanced invasiveness (Klagsbrun *et al.,* 1976; Libermann *et al.,* 1987; Wadzinski *et al.,* 1987; Folkman *et al.,* 1988). In breast tumor cells, FGFR 4 activation results in membrane ruffling, a morphological change that is associated with metastasis (Johnston *et al.,* 1995). In *in vitro* invasion assays, FGF 2 induces the migration of bovine capillary endothelial cells through placental tissue in a dose-dependent manner (Mignatti *et al.,* 1989), and bFGF stimulates production of metalloproteinases in human bladder cancer cell lines, an event associated with increased invasiveness of the cells (Miyake *et al.,* 1997). Moreover,

bFGF-dependent, sustained activation of MAPK correlates with the scattering of neuroepithelioma cells (van Puijenbroek *et al.*, 1997). Together, these studies suggest that FGFs and FGFRs play important roles in human cancer progression by promoting the metastatic process.

D. Platelet-Derived Growth Factor Receptor

The PDGFR has two isoforms, α and β, which differ in their preferences for binding homo- or heterodimers of the A and B forms of the PDGF ligand (Yarden *et al.*, 1986; Claesson-Welsh *et al.*, 1989; Claesson-Welsh and Heldin, 1989; Heldin and Westermark, 1990; Ross *et al.*, 1990; Matsui *et al.*, 1993). Both receptor isoforms consist of an extracellular domain that contains immunoglobulin-like motifs, transmembrane and juxtamembrane regions, a catalytic domain with an insert, and a C-terminal tail (Heldin and Westermark, 1990; Ross *et al.*, 1990). Signaling molecules, which include PI-3 kinase (Kazlauskas and Cooper, 1989; Auger *et al.*, 1989; Coughlin *et al.*, 1989), PLCγ (Kumjian *et al.*, 1989; Meisenhelder *et al.*, 1989; Wahl *et al.*, 1989; Morrison *et al.*, 1990), RasGAP (Molloy *et al.*, 1989; Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990), and the Src family members c-Src, Fyn, and c-Yes (Kypta *et al.*, 1990) (Twamley *et al.*, 1992), bind phosphorylated tyrosine residues in the C-terminal tail, the kinase insert, and the juxtamembrane region via their SH2 domains.

Interestingly, the same downstream effectors in different cell types can elicit different cellular responses. For example, in human hepatoma cell lines, PLCγ and PI-3 kinase can independently transmit mitogenic signals (Valius and Kazlauskas, 1993), whereas in CHO cells a precise balance exists between migration-promoting signaling via PLCγ and PI-3 kinase and migration-inhibitory signaling via RasGAP (Kundra *et al.*, 1994). Phosphorylation of Tyr-988 in the carboxy terminus of the α receptor is associated with induction of chemotaxis, whereas phosphorylation of Tyr-768 and Tyr-1018 negatively regulates this process (Yokote *et al.*, 1996). These results suggest that the different phosphorylation sites serve as binding sites for unique signaling molecules that influence cellular behavior in different ways. This hypothesis is further supported by studies in smooth muscle cells showing that PDGF-induced activation of PLCγ is associated with actin disassembly and chemotaxis, whereas an independent signaling pathway, probably involving small GTPases such as Rho, appears to mediate the proliferative effect of PDGF in this system (Bornfeldt *et al.*, 1995).

PDGF receptors and their ligands regulate a wide spectrum of normal cellular processes in cells of mesenchymal and endothelial origin. These processes include differentiation, proliferation, survival, and migration. For example, the receptor for PDGF α is necessary for the development of neur-

al crest cells (Soriano, 1997) and alveolar branching in the lung (Souza *et al.*, 1995); the PDGF β receptor is required for proper development of the cardiovascular and renal systems (reviewed in Betsholtz, 1995). The PDGF β receptor is also found in mesenchymal tissue of the developing trachea and intestine and in the endothelium of blood vessels, where it is thought to play a role in regulating mesenchymal–epithelial interactions (Shinbrot *et al.*, 1994). In addition, the PDGF α receptor is required for the maximal chemotactic effect of PDGF on lung fibroblasts (Osornio-Vargas *et al.*, 1996).

Numerous studies suggest that various PDGF and PDGFR isoforms are also involved in the genesis or maintenance of human cancers. The PDGFR is overexpressed in human pancreatic cancer (Ebert *et al.*, 1995), primary and metastatic melanomas (Barnhill *et al.*, 1996), and in mesothelioma cell lines (Versnel *et al.*, 1994; Langerak *et al.*, 1996). PDGFR expression is also seen in many neural crest-derived human tumors, including neuroblastoma and Ewing's sarcoma (Matsui *et al.*, 1993), in basal cell carcinoma (Ponten *et al.*, 1994), and in tumors of the lung and pituitary (Leon *et al.*, 1994; Vignaud *et al.*, 1994). PDGF and its receptors are not normally expressed in epithelial cells, but their aberrant expression in tumors of this origin suggest that they could be involved in the oncogenic process.

The situation is made more complex by the fact that some tumors express one or both forms of the ligand and no receptor(s) or vice versa, suggesting that both autocrine and paracrine signaling loops are involved in PDGF-mediated growth of tumors. For example, autocrine signaling loops have been shown to contribute to the growth of human esophageal carcinomas (Juang *et al.*, 1996), mesotheliomas (Langerak *et al.*, 1996), malignant melanomas (Barnhill *et al.*, 1996), gliomas, and glioblastomas (Potapova *et al.*, 1996). However, results from Coltrera *et al.* (1995) show that PDGF may also function in a paracrine fashion in some human breast tumors. Their studies revealed that PDGF β is expressed in breast epithelium and tumor tissues, and the receptor is present in stromal fibroblasts. A similar situation appears to exist in ovarian cancer (Versnel *et al.*, 1994), in lung tumors (Vignaud *et al.*, 1994), and in basal cell carcinomas (Ponten *et al.*, 1994). The ability of PDGF to induce chemotaxis may also play a role in tumor cell metastasis. For example, expression of the receptor for PDGF α in Lewis lung carcinoma cells increases their metastatic potential, whereas expression of the receptor truncated at the kinase domain reverses this effect (Fitzer-Attas *et al.*, 1997). Potapova *et al.* (1996) demonstrated that in human glioblastoma cells, which express both the PDGF β receptor and its ligand, further expression of PDGF β results in tumor formation in nude mice and increased metastasis. These examples support the idea that in addition to mediating normal cell migration, aberrant expression or activation of PDGF receptors in tumor cells can contribute to their proliferative and invasive properties.

E. Epidermal Growth Factor Receptor

The human epidermal growth factor receptor, HER1, belongs to a family of human RTKs that includes HER2/*neu,* HER3, and HER4 (Ullrich and Schlessinger, 1990). All members of this family are transmembrane tyrosine kinases that possess an extracellular domain with two cysteine-rich repeats, an intact catalytic domain, and a C-terminal tail that binds SH2-containing signaling effectors on activation of the receptor. Ligands for HER1 include epidermal growth factor (EGF), transforming growth factor-α (TGF-α), betacellulin (Riese *et al.,* 1996), and epiregulin (Komurasaki *et al.,* 1997). No specific ligand for HER2 has yet been defined, but HER3 and HER4 can be activated by a family of alternatively spliced ligands, called heregulins (HRG) (reviewed in Hynes and Stern, 1994). Each member of the HER family is capable of heterodimerizing with other members of the family, thereby providing a means by which HER2, although it lacks a ligand, can signal. Such dimerization appears to occur in a hierarchical order, wherein the HER2/3 interaction is the most preferred and the HER1/4 interaction the least preferred (Pinkas-Kramarski *et al.,* 1996). Studies in 32D hematopoetic cells, which do not express any HER family members, show that heterodimers have more potent mitogenic activity than do homodimers and that HER3 heterodimers are the most transforming. However, when HER1 is present, signaling through this receptor dominates over other members of the family (Pinkas-Kramarski *et al.,* 1996).

The focus of our discussion will be on the roles of HER1 and HER2 in human cancer, because large bodies of literature exist for each. HER3 and HER4 are more recent additions to the family, and characterization of them with respect to their possible involvement in human cancers is just beginning. However, it is important to note that overexpression of HER3 has been detected in some breast cancers (Lemoine *et al.,* 1992) and in papillary thyroid carcinomas (Faksvag *et al.,* 1996). Thus, the possibility exists that homo- or heterodimerization of HER3 or HER4 with HER1 or HER2 mediates tumorigenic signaling in a manner similar to that of HER1 and HER2.

A major role for HER1 is its involvement in normal human development. It affects many stages, from postfertilization to sexual maturation. For example, HER1 and its ligand, TGF-α, control proliferation of blastocoel cells as well as embryo/uterine signaling and implantation (Rappoll *et al.,* 1988; Dardik and Schultz, 1991; Arnholdt *et al.,* 1991; Zhang *et al.,* 1992). HER1 is also necessary for development of embryonic lung, skin, palate (Lee and Han, 1990), and hair follicles (Hansen *et al.,* 1997). During puberty, HER1 and the estrogen receptor together regulate the differentiation of normal breast epithelium and uterine and vaginal growth (Nelson *et al.,* 1991; Ignar-Trowbridge *et al.,* 1992). Loss of control of these interactions is thought to play a role in the genesis of human tumors, and the diversity of tissues

that are regulated developmentally by HER1 is reflected in the spectrum of tissues and cell types in which HER1 is thought to play an oncogenic role.

That HER1 can function as an oncoprotein was demonstrated by the ability of NIH3T3 cells, engineered to overexpress HER1 and held in the continual presence of EGF, to become transformed and develop tumors in nude mice (Velu *et al.,* 1987; DiFiore *et al.,* 1987a; Di Marco *et al.,* 1989). HER1 effector substrates include the adaptor proteins Shc (Pelicci *et al.,* 1992; Ruff-Jamison *et al.,* 1993) and Grb2, which feed into the well-defined Ras/MAPK signaling pathway (Li *et al.,* 1993; Egan *et al.,* 1993; Rozakis-Adcock *et al.,* 1993), as well as PLCγ (Rhee, 1991), c-Cbl (Levkowitz *et al.,* 1996), eps 8, and eps 15 (Fazioli *et al.,* 1992, 1993a,b). Reports from several laboratories show that on activation, HER1 physically associates with the c-Src nonreceptor tyrosine kinase in both normal fibroblasts and in a variety of tumor cell lines (Luttrell *et al.,* 1994; Maa *et al.,* 1995; Sato *et al.,* 1995; Stover *et al.,* 1995; Biscardi *et al.,* 1998a). Complex formation with c-Src occurs concomitantly with enhanced phosphorylation of receptor substrates, suggesting that c-Src may act to increase the receptor's tyrosine kinase activity, thus enhancing the potential for cellular transformation and tumorigenesis (Maa *et al.,* 1995; Tice *et al.,* 1998; Biscardi *et al.,* 1998a,b). This hypothesis was tested directly using a panel of C3H10T1/2 murine fibroblasts that were engineered to overexpress HER1 and c-Src,-either alone or in combination. Cells overexpressing both HER1 and c-Src were found to produce synergistically larger and more numerous tumors in nude mice and colonies in soft agar than those produced by cells overexpressing either HER1 or c-Src alone (Maa *et al.,* 1995). These findings represent the first causal evidence for cooperativity between c-Src and HER1 in tumorigenesis.

What is the evidence for involvement of HER1 in the genesis of human tumors? Aberrant expression, overexpression, or truncation of HER1 has been demonstrated to occur in a variety of human cancers, including benign skin hyperplasia, glioblastoma, and cancers of the breast, prostate, ovary, liver, bladder, esophagus, larynx, stomach, colon, and lung (Harris *et al.,* 1992; Khazaie *et al.,* 1993; Scambia *et al.,* 1995). In patients with ovarian cancer, overexpression of HER1 correlates with a decreased response to chemotherapy and decreased survival (Scambia *et al.,* 1995; Fischer-Colbrie *et al.,* 1997), suggesting that HER1 plays a proactive role in ovarian tumor progression.

HER1 overexpression also appears to play a role in the etiology of glioblastomas. Forty percent of glioblastomas exhibit amplification of the HER1 gene (Khazaie *et al.,* 1993), but in these tumors, overexpression is not the only abnormality regarding HER1. An alternatively spliced form of the receptor, termed EGFRvIII, is also frequently observed (Libermann *et al.,* 1985; Yamazaki *et al.,* 1988; Tuzi *et al.,* 1991; Chaffanet *et al.,* 1992). This form of the receptor lacks nucleotides 275–1075, which encode a large

portion of the extracellular domain (Humphrey *et al.*, 1990; Ekstrand *et al.*, 1992; Wong *et al.*, 1992, and displays constitutive activity, perhaps due to its inability to be controlled by ligand (Ekstrand *et al.*, 1994). Existing evidence suggests that EGFRvIII signals differently than wild-type receptor, prefering the PI-3 kinase pathway (Moscatello *et al.*, 1998) to the Ras/MAPK pathway (Montgomery *et al.*, 1995; Moscatello *et al.*, 1998). In addition to glial tumors, one study showed that EGFRvIII is present in 16% of non-small-cell lung carcinomas (Garcia *et al.*, 1993) as well as in 86% of medulloblastomas, 78% of breast cancers, and 73% of the ovarian cancers examined (Moscatello *et al.*, 1995). In contrast, EGFRvIII has not yet been detected in normal tissue, a finding that provides compelling evidence for an oncogenic role for this form of the receptor.

A link between HER1 and breast cancer has also emerged in recent years. Amplification or overexpression of the genes encoding one or more of the HER family members is estimated to occur in approximately 67% of human breast cancers (Harris *et al.*, 1992), with overexpression of HER1 detected in approximately 30% of patients (Battaglia *et al.*, 1988; Delarue *et al.*, 1988; Bolla *et al.*, 1990; Koenders *et al.*, 1991; Toi *et al.*, 1991; Harris *et al.*, 1992). Elevated levels of HER1 are also associated with loss of estrogen-dependent growth (Klijn *et al.*, 1993), suggesting a role for HER1 in the later stages of tumor progression.

In addition to transformation and proliferation, studies from several laboratories suggest that HER1 also enhances the invasive potential of tumor cells. Overexpression of HER1 has been shown to result in an increased ability of rat mammary carcinoma cells to migrate through matrigel (Lichtner *et al.*, 1995; Kaufmann *et al.*, 1996), and higher levels of HER1 are found in tumor tissue at metastatic sites as compared to primary sites (Sainsbury *et al.*, 1987; Toi *et al.*, 1991). Both these findings are supportive of a role for HER1 in metastasis.

F. HER2/*neu*

Like HER1, activated HER2 possesses an intracellular tyrosine kinase domain as well as C-terminal phosphotyrosines that are capable of binding downstream substrates, such as PLCγ, PI-3 kinase, Grb7, p120 RasGAP, p190, RhoGAP, c-Src, Shc, PTP1D, PTP1B, eps-8, and Tob, an antiproliferative protein (Hynes and Stern, 1994; Matsuda *et al.*, 1996; Liu and Chernoff, 1997). Because both HER1 and HER2 appear to activate similar downstream signaling pathways in experimental cell systems, it is unclear how specificity of signaling is achieved. The most likely explanation is that activation of a particular signaling pathway is dependent on cell type and on the

subset of HER family members and effector molecules available at any given time. However, a few examples of specific substrates have been reported, such as the c-Cbl adaptor protein for HER1 (Levkowitz *et al.,* 1996) and paxillin and a protein of 23 kDa (p23) for HER2 (Romano *et al.,* 1994).

HER2 is expressed in all tissues except the hematopoietic system (De Potter *et al.,* 1990; Press *et al.,* 1990). Studies using mice that are deficient in HER2, HER4, or the HER3/4 ligand, HRG, demonstrate that signaling through HER2 heterodimers is necessary for proper cardiac and neural development (Meyer and Birchmeier, 1995; Gassmann *et al.,* 1995; Lee *et al.,* 1995). A great deal of evidence from both experimental systems and human patients also points to the involvement of HER2 in malignant transformation. In certain tumors, it has been found that HER2 can be overexpressed up to 100 fold, due to gene amplification (Hynes and Stern, 1994). This finding, coupled with the fact that overexpression of HER2 alone, without the addition of agonist for HER family members, can induce focus formation in cultured fibroblasts (Hudziak *et al.,* 1987; DiFiore *et al.,* 1987b) suggests that overexpression of HER2 is capable of inducing oncogenic activity in the human. In addition, overexpression of HER2/*neu* in PC-3 prostate cancer cells has been shown to result in an increased incidence of metastasis after orthotopic introduction (Zhau *et al.,* 1996). Whereas amplification of the gene encoding HER2 is found in 10–30% of breast, ovarian, and gastric tumors (Hynes and Stern, 1994), tumors of the lung, mesenchyme, bladder, and esophagus contain high levels of HER2 protein but no gene amplification, suggesting that both transcriptional and posttranscriptional mechanisms are responsible for increased HER2 levels (Kraus *et al.,* 1987; Hynes *et al.,* 1989; King *et al.,* 1989; Kameda *et al.,* 1990).

HER2 is apparently involved in the genesis of many types of human tumors, but its role has been most well-characterized in breast cancer. Increased levels of HER2 protein appear to correlate with poor patient prognosis (Slamon *et al.,* 1987, 1989; Paik *et al.,* 1990; Gusterson *et al.,* 1992) and a loss of responsiveness to the antiestrogen, tamoxifen (Nicholson *et al.,* 1990; Wright *et al.,* 1992; Klijn *et al.,* 1993). In transgenic mouse models, HER2/*neu* was demonstrated to induce mammary tumors when expression was targeted to the mammary gland by the use of the murine mammary tumor virus promotor (Muthuswamy *et al.,* 1994). These HER2/*neu* tumors contain increased levels of c-Src and c-Yes kinase activity as compared to normal, surrounding tissue (Muthuswamy *et al.,* 1994; Muthuswamy and Muller, 1995). Furthermore, c-Src was found to coimmunoprecipitate with HER2/*neu* (Muthuswamy *et al.,* 1994), suggesting that c-Src cooperates with HER2 as well as with HER1 in regulating malignant progression. Because HER2/*neu* is most frequently localized to the primary tumor mass in the murine model and is found in earlier stage *in situ* carcinomas in humans

(van de Vijver *et al.*, 1988; Paik *et al.*, 1990; Lin *et al.*, 1992; Barnes *et al.*, 1992), it is speculated that this molecule is involved in earlier stages of breast cancer than is HER1.

G. HER Family Members and Estrogen Receptor Interactions

Increasingly compelling data are accumulating that point to interactions among the estrogen receptor (ER), HER1, HER2, and c-Src as being major factors in the development of human breast cancer. The ER is a steroid hormone receptor of 67 kDa that dimerizes and becomes activated as a transcription factor on binding of estrogen (Mangelsdorf *et al.*, 1995). Functional domains of the ER include an amino-terminal A/B region, which is responsible for ligand-independent transcriptional activation; a central DNA-binding domain; and a carboxy-terminal E/F hormone-binding domain, which is responsible for estradiol-induced transcription (Tsai and O'Malley, 1994; Beato *et al.*, 1995). In addition to the well-characterized ER α isoform, a β isoform, which has differing transcriptional properties and expression patterns, has been discovered (Paech *et al.*, 1997).

Loss of ER responsiveness in human breast tumors correlates with overexpression of HER1 and with a poorer patient prognosis (Fitzpatrick *et al.*, 1984; Sainsbury *et al., 1985; Davidson et al.*, 1987; Nicholson *et al.*, 1988). The mechanism by which a breast tumor cell loses responsiveness to estrogen is unclear, but this event may be regulated in part by interactions with HER family members and/or c-Src. Cross-talk between growth factor receptor tyrosine kinases and the ER was first demonstrated by Ignar-Trowbridge and co-workers (1992, 1993), who showed that treatment of cells with EGF activates the transcriptional activity of the ER and that this effect is dependent on the amino-terminal A/B domain of the ER. The ER also appears to have the ability to affect expression of the EGF receptor. In ER-positive breast cancer cells, estradiol treatment increases HER1 mRNA levels (Yarden *et al.*, 1996). This effect may be directly mediated by the ER, because the HER1 promoter has sequences that share loose homology with the estrogen response element (ERE) and can bind human ER (Yarden *et al.*, 1996).

Conversely, HER1 can affect ER expression. Overexpression of TGF-α in the ER-positive ZR75-1 breast cancer cell line, along with prolonged treatment of these cells with antiestrogens, results in loss of the ER, whereas treatment of parental ZR75-1 cells with antiestrogens alone has little effect (Clarke *et al.*, 1989; Agthoven *et al.*, 1992). These results are interpreted to mean that continual and concomitant stimulation of HER1 and ER can cause a reduction in ER expression. In this regard, it is through that expres-

sion of HER1 and ER and mutually exclusive, because most breast tumors that overexpress HER1 lack functional ER (Fitzpatrick *et al.,* 1984; Sainsbury *et al.,* 1985; Davidson *et al.,* 1987; Nicholson *et al.,* 1988). Because breast tumors that do not show this inverse expression tend to be HER1/ER positive rather than HER1/ER negative, it has been suggested that overexpression of HER1 precedes loss of the ER (Koenders *et al.,* 1991; Dittadi *et al.,* 1993; Chrysogelos and Dickson, 1994).

It is unclear how overexpression or activation of HER1 leads to loss of ER expression. One possible mechanism may involve signaling to MAP kinase. HER1 activation results in the phosphorylation of the ER on Ser-118, a phosphorylation that is required for hormone-independent transcriptional activity of the ER (Kato *et al.,* 1995). Ser-118 is also thought to be a target for MAP kinase, because studies using dominant negative Ras and MEK demonstrated a loss of this phosphorylation concomitantly with a loss of EGF-dependent transcriptional activation (Bunone *et al.,* 1996). Autocrine stimulatory loops involving TGF-α and HER1 are known to exist in breast cancer, thus it is speculated that the continued stimulation of the ER via the HER1/MAP kinase pathway leads to its down-regulation and eventual loss.

Estradiol is also known to induce phosphorylation of the ER (Auricchio *et al.,* 1987). In addition, Arnold *et al.* (1995a) reported that the ER is basally phosphorylated on Y537 *in vivo.* The role of the Y537 phosphorylation is controversial. Early studies showed that tyrosine phosphorylation of the ER activates its hormone-binding activity (Migliaccio *et al.,* 1989) and that phosphorylation of Y537 is required for binding of the ER to the ERE (Arnold and Notides, 1995; Arnold *et al.,* 1995b). Further *in vitro* studies demonstrated that c-Src is able to phosphorylate Y537 and that this phosphorylation is necessary for homodimerization of the ER and for binding of estradiol (Arnold *et al.,* 1995a, 1997). In agreement with these findings, Castoria *et al.* (1996) reported that a non-hormone-binding form of the ER found in mammary tumors can be converted to a hormone-binding form by *in vitro* phosphorylation with a calcium/calmodulin-regulated kinase, which is thought to be a c-Src family member. However, additional studies in which Y537 was mutated to various amino acids suggest that phosphorylation of Y537 per se is unnecessary for estradiol-mediated activation of the ER but may be important in ligand-independent (i.e., growth factor-mediated) activation (Weis *et al.,* 1996; Lazennec *et al.,* 1997).

Although c-Src is capable of phosphorylating the ER, the ER may also influence c-Src activity. Estradiol treatment has been shown to increase c-Src tyrosine phosphorylation and kinase activity in MCF7 breast cancer cells (Migliaccio *et al.,* 1993, 1996) and to stimulate kinase activity of c-Src and its related family member, c-Yes, in colon carcinoma cells (Di Domenico *et al.,* 1996).

Ligand-independent down-regulation of the ER may also be mediated by

HER2 signaling pathways. Pietras *et al.* (1995) showed that overexpression of HER2 in MCF7 cells leads to estrogen-independent growth and ERE transcriptional activation. Furthermore, treatment of these cells with HRG, which stimulates HER2-dependent signaling via HER2/3 heterodimers, induces tyrosine phosphorylation and down-regulation of the ER. Other investigators have shown that HRG treatment inhibits the expression of ER in ER-positive breast cancer cells and can revert the estradiol-mediated decrease in HER2 expression (Grunt *et al.*, 1995). Taken together, these results suggest that in experimental cell systems, HER2 and the ER are expressed in a mutually exclusive manner. However, in human breast tumors, the situation is less clear, with some reports indicating an inverse correlation between HER2 and ER expression (Adnane *et al.*, 1989; Borg *et al.*, 1990) and others indicting no such correlation (Slamon *et al.*, 1989; Bacus *et al.*, 1996).

III. c-Src AND c-Src FAMILY MEMBERS IN HUMAN CANCERS

A. c-Src Structure and Mechanisms of Regulation

c-Src is the cellular, nontransforming homolog of v-Src, the oncoprotein encoded by the chicken retrovirus, Rous sarcoma virus. c-Src is a 60-kDa tyrosine kinase that is composed of six domains: an N-terminal membrane-association domain, a "Unique" domain, SH3 and SH2 domains, a catalytic domain, and a negative regulatory domain (Fig. 2, see color plate). Although c-Src is cytosolic, it localizes to intracellular membranes, including the plasma membrane and membranes of endosomes and secretory vesicles within the cytosol (Parsons and Creutz, 1986; Kaplan *et al.*, 1992; Resh, 1994). It is tethered to these membranes by the combined action of an N-terminal, covalently linked myristate moiety, salt bridges between basic amino acids in the N terminus and phosphates of the lipid backbone, and noncovalent interactions with integral or associated membrane proteins (Resh, 1994). Membrane localization of c-Src is required for its ability to participate in growth factor receptor-mediated signaling in normal cells (Wilson *et al.*, 1989). The function of the Unique domain is not well-defined. However, based on the fact that it exhibits the greatest sequence divergence among family members of all the domains (Brown and Cooper, 1996), it is speculated to specify protein–protein interactions that are unique to individual Src family members. The SH3 and SH2 domains mediate the binding of c-Src with other signaling proteins through proline-rich or phosphotyrosine-containing regions on target proteins, respectively (Pawson and Schlessinger, 1993). The major regulatory region of the enzyme is a short domain at the extreme C terminus of

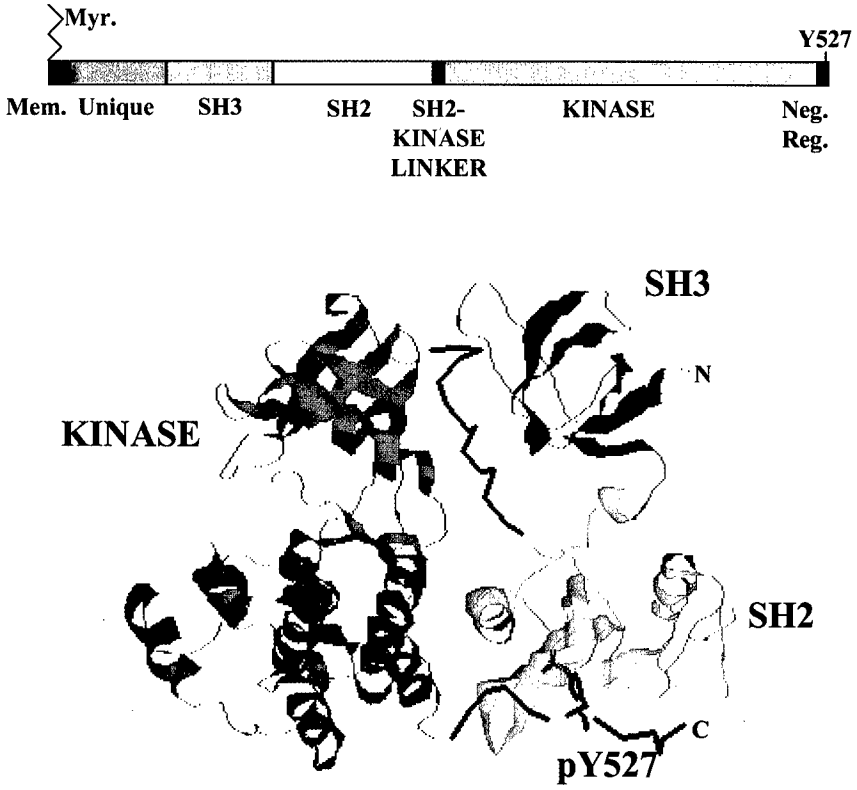


Fig. 2 Structure of c-Src. c-Src is the prototype of a large family of cytoplasmic tyrosine kinases that associate with cellular membranes through lipid modifications at their N termini. As a linear molecule, the relationship between the various domains can be seen: an N-terminal membrane association domain that contains the site of myristylation, a Unique domain that exhibits the widest sequence divergence among family members of any of the domains, an Src-homology-3 (SH3) domain that binds poly(proline) motifs on target molecules, an Src-homology-2 (SH2) domain that binds phosphotyrosine residues on target molecules, an SH2/kinase linker, the catalytic domain, and the negative regulatory domain that contains the predominant site of tyrosine phosphorylation on the inactive molecule (Y527 in chicken, Y531 in human). The three-dimensional orientation of the molecule, lacking the membrane-association and Unique domains, is depicted as a ribbon diagram. Reprinted with permission from *Nature,* Xu *et al.* (1997), and from Michael Eck (configured from the atomic coordinates provided on the Web). Copyright 1997 Macmillan Magazines Limited. The enzymatic activity of c-Src is regulated by the coordinated effects of target proteins binding to or covalent, post-translational modifications of the SH3, SH2, and negative regulatory domains on the catalytic domain, as described in the text.

the molecule, which harbors a Tyr residue that becomes phosphorylated (Y530 in human c-Src; Y527 in chicken c-Src) by a C-terminal Src kinase, CSK (Okada *et al.,* 1991). Phosphorylated Y527/530 (pY527) is capable of binding its own SH2 domain in a manner that inhibits kinase activity without physically blocking the catalytic site, as shown in Fig. 2 (Yamaguchi and Hendrickson, 1996; Sicheri *et al.,* 1997; Xu *et al.,* 1997).

Binding of tyrosine-phosphorylated cellular proteins to the SH2 domain is thought to destabilize the intramolecular pY527/SH2 domain interaction and induce a conformational change that results in enzymatic activation. Structural studies have revealed that the SH2 and SH3 domains collaborate in their binding of respective protein partners, thereby cooperatively influencing the activity of the enzyme (Eck *et al.,* 1994). Furthermore, crystallographic analysis has shown that sequences just N terminal to the catalytic domain (termed the SH2-kinase linker) comprises a loop structure that functions as a "pseudo" SH3 binding site (Yamaguchi and Hendrickson, 1996; Sicheri *et al.,* 1997; Xu *et al.,* 1997). Together, the intramolecular phosphotyrosine/SH2 and linker/SH3 interactions direct a conformation that presses the linker against the backbone of the catalytic domain and renders the protein inactive. As with the SH2 domain, binding of signaling proteins to the SH3 domain is thought to release the constraints of the linker/SH3 interaction on the kinase domain, resulting in activation of catalytic activity.

Mutation of Y527 to F or deletion of the C-terminal regulatory domain (as in v-Src) results in a constitutively active protein that phosphorylates target proteins in an unregulated fashion and induces cellular transformation and oncogenesis (Cartwright *et al.,* 1987; Kmiecik and Shalloway, 1987; Piwnica-Worms *et al.,* 1987; Reynolds *et al.,* 1987). In normal cells, c-Src is nononcogenic or only weakly so, even when it is overexpressed (Shalloway *et al.,* 1984; Luttrell *et al.,* 1988). However, under certain conditions (growth factor stimulation or translocation; outlined below), the enzyme can become activated, either via dephosphorylation of pY527 or by binding of signaling proteins to the N-terminal half of the protein. Activation is most frequently a transient event, and c-Src, in contrast to v-Src, is thought to respond to negative control by rephosphorylation of Y527 or by the release of binding proteins and the resumption of intramolecular interactions. It has been the conjecture of many investigators that the transient nature of c-Src activation often prevents its detection. In fact, the possibility exists that little or no activation above basal levels is necessary for catalysis, if the substrate is properly positioned near the catalytic cleft. Thus, another "regulator" of c-Src activity may well be its intracellular localization and, at a finer level, its appropriate juxtaposition to substrate within a signaling complex. Identification of c-Src substrates and proteins that bind its SH2 and SH3 domains is now critical for further understanding of the role c-Src and its family members play in biological processes.

The majority of the studies leading to the above model have been conducted in animal tissue culture systems and are just now being applied to the study of c-Src in human tumors. In the following section evidence is presented for the involvement of c-Src in the genesis of human tumors, with particular emphasis on its putative role in colon, breast, lung, and myeloid tumors.

B. Evidence for the Involvement of c-Src in Human Cancers

Like the RTKs, many lines of evidence are suggestive of a role for c-Src in the genesis and progression of multiple types of human cancer. This evidence is both genetic and biochemical in nature and has been generated by studies of cultured tumor cell lines and surgically generated tumor tissue. Together these studies have implicated c-Src as an etiological agent for the development of neuroblastomas, myeloproliferative disorders (including myeloid leukemia), and carcinomas of the colon, breast, lung, esophagus, skin, parotid, cervix, and gastric tissues. Interestingly, although alterations of c-Src have been described at both the gene and protein levels in various cancer tissues, the changes are quite variable and include both increases and decreases in gene copy number and in protein levels and specific enzyme activities. Taken at face value, these findings suggest multiple ways in which c-Src can contribute to the oncogenic process, both as a dominantly acting oncogenic protein and as a negatively acting tumor suppressor. However, the multitude of changes could also reflect fortuitous alterations that do not contribute to the ultimate malignant phenotype. There may also be technical reasons for the variability in the findings, such as the different probes used for genetic analysis and the different antibodies and cell extraction conditions used for biochemical analysis. It is clear that further work needs to be done to clarify these issues and attempts made to minimize technical problems. Of particular importance to future studies will be the development and characterization of good animal and tissue culture models to test the hypotheses derived from analyses of human tumor tissues, whereby the contribution of individual genes or proteins can be evaluated for their oncogenic potential against a normal cell background rather than against a heterogeneous background of unknown numbers and types of genetic alterations that occur in every human tumor.

1. GENETIC EVIDENCE

With the identification of the first protooncogenes came a plethora of studies examining the genomic content of multiple human tumors for deletions, amplifications, and diverse rearrangements in chromosomes containing pro-

tooncogenes. For the most part, these studies identified few if any gross changes in the c-Src gene, which maps to the q arm of chromosome 20. Furthermore, gene expression studies, employing a variety of techniques to measure steady-state levels and newly synthesized mRNA have also revealed few changes in c-Src-specific mRNA (Bishop, 1983; Slamon *et al.*, 1984). These findings led many investigators to conclude that c-Src played a minor (if any) role in the genesis of human tumors. Not until researchers began examining protein levels and specific enzyme activities did evidence for the involvement of c-Src begin to emerge.

However, there were a few exceptions to the general rule described above, and one in particular is noteworthy. Four groups have identified a deletion of 16–21 cM in the long arm of chromosome 20 [del(20q)] as a recurring, nonrandom abnormality in malignant myeloid disorders, including nonlymphocytic leukemia and polycythemia (Simpson, 1988; Roulston *et al.*, 1993; Hollings, 1994; Asimakopolous *et al.*, 1994). This deletion maps between 20q11.2 and 20q13.3, a region that encodes the c-Src protooncogene (Hollings, 1994). The notion that deletion of a chromosomal region is signatory for a tumor suppressor gene suggests that, if c-Src is a critical gene in this deletion, it behaves as a negative regulator of cell growth, not as a dominant oncogene, as is commonly believed. That c-Src may have some tumor suppressor-like characteristics in myeloid cells is supported by the finding of several groups (Barnekow and Gessler, 1986; Gee *et al.*, 1986) that c-Src expression levels increase during myeloid differentiation. If c-Src plays a critical role in promoting differentiation and maintaining the postmitotic state, then loss of such an activity might permit cells to once again acquire proliferative activity—the hallmark of a tumor suppressor.

2. BIOCHEMICAL EVIDENCE

By far the bulk of evidence supporting a role for c-Src in the development of human tumors comes from biochemical studies, wherein the levels of c-Src protein and tyrosine kinase activity have been examined in hundreds of human tumors and compared to normal tissue controls. As will be discussed in more detail below, in some tumor specimens, high enzymatic activity is accompanied by high protein level, yielding little or no change in specific activities, whereas in others, protein levels are only slightly or modestly elevated, and the specific activity of the enzyme is increased. In yet other examples, high protein levels are accompanied by low enzymatic activity. However, the overall conclusion is that in a very high percentage (>50% and approaching 100% in some studies) of human tumors of many different tissue types, c-Src activity is altered (usually elevated) and that this alteration occurs in early to middle stages of tumor progression and is maintained or increased throughout progression to metastasis.

These findings raise questions as to the mechanism of c-Src activation and the mechanisms by which protein levels are elevated (especially in light of the few instances of increases in c-Src-specific mRNA production). The consensus at the present time is that changes in c-Src specific activity in human tumors are due to posttranslational events and not to mutations of the gene. Using RNase protection and restriction fragment-length polymorphism assays to detect activating mutations of c-Src in a spectrum of human tumors, Wang *et al.* (1991) were unable to detect mutations at codons known to contribute to the oncogenicity of v-Src and c-Src (namely, codons 98, 381, 444, and 530 in the human c-Src sequence). These findings led the investigators to conclude that mutational activation is not the mechanism of enhancement of c-Src-specific kinase activity. On the other hand, DeSeau *et al.* (1987) described differential activation of c-Src in normal colon cells versus colonic tumor cells depending on the conditions of extract preparation, i.e., whether the lysis buffers contained the proteins tyrosine phosphatase inhibitor, vanadate, and/or high concentrations of ionic and nonionic detergents. From these results, one could deduce that tyrosine phosphorylation of c-Src or other cellular proteins and protein/protein interactions play a role in regulating not only c-Src activity but also its stability and abundance. Indeed, structural studies on the c-Src molecule described above would support this notion. However, so as not to think that the issue is resolved, studies by Watanabe *et al.* (1995) indicate that in 18 cancer cell lines, elevated activities of c-Src and c-Yes (a Src-related family member) are accompanied by correspondingly elevated levels of C-terminal Src kinase, the protein that phosphorylates Y530 in human c-Src and negatively regulates c-Src kinase activity. These findings suggest that CSK may not have an antioncogenic role to play in tumor progression or that dephosphorylation of Y530 is not required for activation of c-Src.

Here the focus is on three different carcinomas—colon, breast, and lung—for which substantial amounts of data are accumulating to indicate a role for c-Src in their development. That these represent three of the four most common forms of cancer in adults (prostate cancer being the fourth) suggests that c-Src may be a more formidable player in tumorigenesis than had previously been appreciated.

3. COLON CANCER

Utilizing c-Src-specific antibodies and an immune complex-based tyrosine kinase assay, a number of investigators have reported that c-Src-specific tyrosine kinase activity (total activity relative to total c-Src protein in an immune complex) is elevated in colon cancer. In panels of colon cancers examined by Rosen *et al.* (1986), Bolen *et al.* (1987a,b), and Cartwright *et al.*

(1989), c-Src was found to exhibit elevated kinase activity, ranging from ~2- to 40-fold above that found in normal colon tissues or cultures of normal colon mucosal cells. In some cases this increase could be accounted for by increases in protein levels, but in other instances it could not, indicating an increase in specific kinase activity. These results suggest that either elevation in c-Src protein and/or activation of c-Src may contribute to the genesis of human colon tumors. Indeed, additional studies by Lundy *et al.* (1988) and Cartwright *et al.* (1990, 1994) demonstrated increased kinase activity in premalignant epithelia of ulcerative colitis and in early-stage colonic polyps as compared to adjacent normal mucosa. In the latter study, activity was highest in malignant polyps and in >2-cm benign polyps that contained villous structure and severe dysplasia. Thus, c-Src activity is found to be elevated in early stages of colon cancer and this elevation is associated with those polyps that are at greatest risk for developing cancer. Talamonti *et al.* (1993) also demonstrated incremental increases in c-Src activity and protein level as the tumors progressed, with the greatest increases seen in metastatic lesions. Increases in specific kinase activity were also observed, with liver metastases exhibiting an average increase of 2.2-fold over normal mucosa, whereas extrahepatic metastases demonstrated an average 12.7-fold increase. These results support the idea that c-Src may play multiple roles in tumor progression.

A number of studies have been done to determine if c-Src indeed plays a causal role in tumor development. Herbimycin A, an inhibitor of Src family kinases, was shown to inhibit the growth in monolayer of seven colon tumor cell lines as compared to one cell line from normal colonic mucosa, CCL239 (Garcia *et al.*, 1991). In another study, blockage of the myristylation modification of Src family members in a panel of human colon adenocarcinoma tumor cell lines by *N*-fatty acyl glycinal compounds was shown to prevent localization of c-Src to the plasma membrane and to depress colony formation of these cell lines in soft agar and cell proliferation assays (Shoji *et al.*, 1990). Tumor necrosis factor (TNF-α-mediated growth inhibition of human colorectal carcinoma cell lines was accompanied by a reduction in the activity of s-Src (Novotny-Smith and Gallick, 1992). And last, using an antisense expression vector specific for c-Src, Staley *et al.* (1997) demonstrated that expression of c-Src antisense in HT29 human colon adenocarcinoma cells resulted in slower proliferation and slower growing tumors in nude mice as compared to the parental control. Together, these studies are consistent with a causative role for c-Src in colon cancer progression.

How could c-Src be functioning to promote progression of colonic tumors? Using an *in vitro* progression model based on the PC/AA premalignant colonic adenoma cell line, Brunton *et al.* (1997) demonstrated that in the conversion from adenoma to carcinoma, levels of both the EGF receptor and FAK protein increased, while the expression and activity of c-Src were

unaltered. However, EGF induced motility in the carcinoma cells, but not in the adenoma cells, and this increase was accompanied by an EGF-induced increase in c-Src kinase activity, relocalization of c-Src to the cell periphery, and phosphorylation of FAK. The authors interpret these findings to indicate that c-Src is not the driving force for tumor progression, but cooperates with other molecules (such as EGFR and FAK) in the process. Other investigators have observed that adhesion of HT29 human colon carcinoma cells to E-selectin results in a decrease in c-Src activity (Soltesz *et al.*, 1997), suggesting that, on release from substratum restrictions, c-Src activity is restored or elevated. In a related study, Empereur *et al.* (1997) generated evidence for cooperativity between c-Src and HGF/SF in developing invasive properties of the PC/AA cell line. Specifically, introduction of activated c-Src or polyoma middle-T antigen (which requires c-Src for oncogenic activity) into the adenoma PC/AA cell line induced conversion of the adenoma to carcinoma, overexpression of the HGF receptor, and an invasive capacity in the presence of HGF. Thus, current evidence suggests that one mechanism by which c-Src promotes colonic tumor progression is by cooperating with components of the cell adhesion/motility machinery. Similar conclusions were reached by Mao *et al.* (1997), who demonstrated activation of c-Src in response to EGF or HGF treatment of human colon cancer cells with high metastatic potential.

4. BREAST CANCER

As with colon cancer, a number of early investigations reported elevated c-Src activity in human breast cancers (Jacobs and Rubsamen, 1983; Rosen *et al.*, 1986; Lehrer *et al.*, 1989). In several reports the elevation in activity was not accompanied by elevated levels of c-Src protein, suggesting an activation of the protein. However, Koster *et al.* (1991), using a screening method based on *in vitro* synthesis of cDNA copied from total cellular RNA of tumor tissue, found that 25–30% of the analyzed tumors showed significant elevations in expression of several protooncogenes, including c-Src. Using immune complex kinase assays, immunoblotting, and immunohistochemical approaches, Verbeek *et al.* (1996) and Biscardi *et al.* (1998a) demonstrated that increases in c-Src kinase activity are almost invariably accompanied by increases in c-Src protein levels and little if any change in specific kinase activity. Interestingly, the immunohistochemical studies of Verbeek *et al.* (1996) showed that in malignant cells, the majority of c-Src appeared to be concentrated around the nucleus, whereas in normal cells, it is distributed more evenly in the cytoplasm. The discrepancies between the more recent data and the earlier data may reflect changes in the quality of the antibodies and the more quantitative analyses performed in the recent studies. In total, the current evidence indicates that few "activations" of c-

Src occur in breast tumor cells; rather, elevations in protein levels appear to be the major cause of the increases in c-Src kinase activity. In a recent study involving 72 breast cell lines and tumor biopsies, tyrosine kinase activity was found to be elevated in 100% of the samples, as compared to normal tissue controls, and c-Src tyrosine kinase accounted for 70% of the total cytosolic activity (Ottenhoff-Kalff *et al.*, 1992). The same group performing that study had previously found that the level of cytosolic protein tyrosine kinase activity parallels the malignancy in breast tumors (Hennipman *et al.*, 1989) and that the majority of this activity is precipitated by anti-c-Src antibodies. These results provide compelling correlative evidence that c-Src plays a key role in the development of breast cancer. In agreement with this conclusion, Lehrer *et al.* (1989) and Koster *et al.* (1991) also note that elevated c-Src kinase activity is most frequently found in tumors that are progesterone receptor negative. Because loss of progesterone receptor is a histochemical marker for later stage tumors, c-Src activity appears to increase as the tumor progresses in severity.

To directly assess the effect of mammary gland-specific expression of c-Src, Webster *et al.* (1995) established transgenic mice that carried a constitutively activated form of c-Src under the transcriptional control of the murine mammary tumor virus long terminal repeat. Female transgenic mice exhibited a lactation defect and frequently developed mammary epithelial hyperplasias, which occasionally progressed to frank neoplasias. The authors interpret these results to mean that expression of activated c-Src in the mammary gland is not sufficient for induction of mammary tumors—that some other event must take place for frank neoplasias to occur. That c-Src can play more than a bystander role in tumor development, however, was demonstrated by the experiments of Guy *et al.*, (1994), wherein mice transgenic for the polyoma virus middle-T antigen under the control of the murine mammary tumor virus long terminal repeat developed tumors when in a genetic background positive for c-Src, but not when in a background null for c-Src. Similar results were obtained by Amini *et al.* (1986a), who used c-Src antisense expression vectors to demonstrate that c-Src is required for transformation of rat FR3T3 cells by polyoma middle-T antigen in tissue culture. Together, these studies indicate that c-Src is necessary but not sufficient for tumor development in the mammary gland.

5. LUNG CANCER

Lung cancer is the leading cause of cancer death in the United States. Small cell lung cancer (SCLC) accounts for 20–25% of all bronchogenic carcinoma and is associated with the poorest 5-year survival of all histologic types. c-Src expression was found to be elevated in 60% of all lung cancers (Mazurenko *et al.*, 1991b), when biopsy material of tumors, metastases, and

"normal" surrounding tissues from patients with different histological types of stomach and lung cancer, melanoma, and other malignancies were analyzed by immunoblotting and immunohistochemistry. A breakdown of the lung histologic types exhibiting increased c-Src expression revealed that c-Src protein was elevated in SCLC and atypical carcinoid tumors, as well as in non-small-cell tumors, such as adenocarcinoma, bronchoalveolar, and squamous cell lung cancer (Mazurenko *et al.*, 1991a). In these studies no analysis of c-Src kinase activity was reported. Somewhat contrasting results were reported by authors of a study in which 60 human cell lines used by the National Cancer Institute for the random screening of potential anticancer drugs were analyzed for c-Src kinase activity. In this study SCLC-derived cell lines had a low activity, whereas non-small-cell lung tumors exhibited activity that was greater than that observed in colon cancer cells, which are considered to have high c-Src activity (Budde *et al.*, 1994). The findings from these studies are strongly supportive of other investigations, concluding that c-Src is frequently overexpressed in SCLC and other types of lung cancer (Cook *et al.*, 1993).

6. OTHER CANCERS

Many other tumor types exhibit elevations in c-Src kinase activity or protein/mRNA levels, including neuroblastomas (Bjelfman *et al.*, 1990) and carcinomas of the esophagus (Jankowski *et al.*, 1992; Kumble *et al.*, 1997), gastric tract (Takekura *et al.*, 1990), parotid gland (Bu *et al.*, 1996), ovary (Budde *et al.*, 1994), and skin (Kim *et al.*, 1991). With regard to skin cancers, a study carried out in a mouse model of epidermal tumor promotion described activation of erbB2 and c-Src in phorbol ester-treated mouse skin as a possible mechanism by which phorbol esters promote skin tumors in mice. Activation of erbB2 and c-Src kinase is also observed in the epidermis of TGFα transgenic mice, where expression of human TGFα was targeted to basal keratinocytes (Xian *et al.*, 1997). In cervical cancer, evidence is beginning to emerge for cis activation of cellular protooncogenes (including c-Src) by integration of human papillomavirus DNA into the genome of cervical epidermal cells (Durst *et al.*, 1987). In tissue culture studies using primary hamster embryo cells, infection with other DNA tumor viruses, such as SV40, adenovirus, or bovine papillomavirus, also results in increases in the specific activity of c-Src (Amini *et al.*, 1986b).

C. c-Src Family Members and Human Cancers

c-Src is the prototype for a family of nonreceptor protein tyrosine kinases, for which novel members are regularly being identified. Current members

include c-Src, Fyn, c-Yes, Lck, Hck, Lyn, c-Fgr, Blk, and Yrk (Brickell, 1992; Sudol *et al.,* 1993; Brown and Cooper, 1996). All members have the same overall structure and minimally contain Unique, SH3, SH2, and kinase domains. The greatest sequence divergence occurs in the Unique domain, thus its name. Not all members are linked to lipids at the N terminus, nor are all negatively regulated by a C-terminal domain that includes the Tyr-530 homolog of human c-Src. c-Fgr, Lck, Hck, and Blk are expressed predominantly in cells of hematopoietic lineage, whereas c-Src, c-Yes, Fyn, Lyn, and Yrk are more ubiquitous. All members have been implicated in various signal transduction pathways, and with the mounting evidence for involvement of c-Src in the genesis of multiple human cancers, the question arises as to whether close relatives of c-Src may also be implicated in these diseases. If so, there are other questions that warrant investigation: Is more than one family member involved in the genesis of the same tumor type? Do c-Src family members fulfill overlapping or unique functions in promoting tumor formation and progression? Are there members of this family that are expressed exclusively in tumors as compared to normal tissue? A review of the literature reveals a paucity of information with regard to any of these issues. It is not clear whether this paucity reflects the unavailability of useful and appropriate reagents to investigate the questions, or whether studies have been conducted and few have uncovered evidence for c-Src family member involvement. Although the following description is not meant to be comprehensive, it does suggest that family members in addition to c-Src may be involved in the genesis of human and certain animal tumors.

1. GENETIC EVIDENCE

In humans, sequences related to the human c-Yes gene were found to be amplified in a single primary gastric cancer out of 22 cases that were examined (Seki *et al.,* 1985). The sequences were amplified four- to fivefold, but normal stomach tissue adjacent to the tumor tissue in the same patient showed no amplification. In the dog, a protooncogene related to the human c-Yes gene was detected as restriction fragment-length polymorphisms (RFLPs) in a Southern blot analysis of genomic DNA from six canine primary mammary tumors in a screen employing seven protooncogene probes. These RFLPs were 0.1 to 1.0 kb shorter than the normal gene, suggesting the occurrence of chromosomal rearrangements and possible deregulation of gene expression, leading to tumorigenesis (Miyoshi *et al.,* 1991). Melanoma formation in the fish *Xiphophorus* is a genetic model for the function of tyrosine kinases in tumor development. In malignant melanomas from these fish, elevated levels of c-Yes and Fyn activity have been detected as compared to normal tissue (Hannig *et al.,* 1991). Fyn has also been found to coprecipitate with the *Xiphophorus* melanoma receptor kinase (Xmrk), the molecule

that is responsible for the formation of hereditary malignant melanoma in this lower vertebrate (Wellbrook *et al.*, 1995). These results suggest that Xmrk may function at least in part through Fyn in melanoma formation.

2. BIOCHEMICAL EVIDENCE

In studies similar to those conducted for c-Src, evidence for the involvement of other c-Src family members in the etiology of human cancers is emerging, but at a much slower pace than that for c-Src. Elevated c-Yes tyrosine kinase activity has been detected in premalignant lesions of the colon that are at greatest risk for developing cancer (Pena *et al.*, 1995). In this study, the activity of c-Yes in such adenomas was 12- to 14-fold greater than activity in adjacent normal mucosa. Similar results were obtained when mRNA levels of nine protooncogenes in colonic tissue from patients with inflammatory bowel disease (IBD) were measured. The steady-state level of c-Yes-encoded mRNA was considerably higher in IBD patients resected for colon cancer than in patients resected for active chronic IBD or in controls (Alexander *et al.*, 1996). These results suggest that expression of this gene may be a marker for development of colon cancer in IBD. Finally, in rodents, the action of the transforming proteins of mouse and hamster polyomaviruses (middle-T antigens) is mediated in part through c-Src family kinases, with preferential action of hamster T antigen for Fyn (Brizuela *et al.*, 1995).

c-Src family members have also been implicated in the genesis of diseases involving Epstein–Barr Virus (EBV), such as Burkitt's lymphoma, Hodgkin's disease, and nasopharyngeal cancer. All of these diseases involve abnormal proliferation of B cells. EBV encodes two transformation-associated proteins, LMP1 and LMP2, that are integral membrane proteins. LMP2 mRNA is the only EBV-specific message detected in B lymphocytes from individuals harboring EBV latent infections. LMP2 protein also associates with c-Src family tyrosine kinases, LMP1, and other unidentified proteins, suggesting that the association of these two EBV-encoded membrane proteins could create a macromolecular complex mediating constitutive B lymphocyte activation through normal cell signal transduction pathways (Longnecker, 1994).

In human malignant melanoma and other cancers, aberrant expression of basic fibroblast growth factor (bFGF) causes constitutive autocrine activation of its cognate receptor and autonomous growth of tumor cells in culture (see above). Expression of a dominant-negative mutant of the FGF receptor (lacking the kinase domain) was found to suppress tumor formation in nude mice and markedly reduce c-Src family kinase activity in melanoma cells (Yayon *et al.*, 1997). Together these studies suggest that c-Src family kinases play an important role in maintenance and/or progression of malignant melanoma.

D. Nonreceptor Tyrosine Kinases Related to c-Src Family Members and Human Cancers

Several new nonreceptor tyrosine kinases have been isolated from human breast cancer cells. A cDNA encoding a 54-kDa phosphoprotein, called Rak, was cloned from human breast cancer cells (Cance *et al.*, 1994). This protein shares 51% identify with c-Src and contains SH3, SH2, kinase, and negative regulatory domains. However, it has some properties that are distinct from c-Src, such as its predominant expression in epithelial cells, its lack of a myristylation site, and its almost exclusive localization to the nucleus. However, like c-Src, Rak is overexpressed in subsets of primary human epithelial tumors, suggesting that it may play a role in development of human cancer. Another protein, named Brk (breast tumor kinase), appears to be expressed exclusively in breast tumor tissue as opposed to normal mammary epithelium (Barker *et al.*, 1997). Approximately two-thirds of breast tumors express appreciable levels, and 27% of these overexpress Brk 5- to 40-fold or more. When overexpressed in fibroblasts or mammary epithelial cells, Brk sensitizes cells to the action of EGF and also induces a partial transformed phenotype (Kamalati *et al.*, 1996). These findings suggest that Brk is a functionally important factor in the evolution of breast cancer.

IV. MECHANISMS OF c-Src ACTION

A. Evidence for Involvement of c-Src in Signaling through Receptor Tyrosine Kinases

The elevated levels of c-Src expression and/or activation in a wide spectrum of human tumors suggest that c-Src is contributing in some way to the neoplastic phenotype. That c-Src is overexpressed in many of the same tumors in which specific RTKs are also often overexpressed suggests that the two classes of tyrosine kinases may functionally interact to promote tumorigenesis. Many of the RTKs are oncogenic when overexpressed or inappropriately expressed, as described above. The question that follows is whether c-Src is required for the oncogenic capabilities of overexpressed RTKs, or whether c-Src enhances or contributes to RTK-mediated oncogenesis by any means. This latter question was in part addressed when it was shown that cooverexpression of c-Src with HER1 in a mouse fibroblast model resulted in synergistic increases in tumor volume, as compared to tumors developed by cells overexpressing only one of the pair of kinases (Maa *et al.*, 1995). These results provided direct evidence for the enhancing effect of c-Src on receptor-transforming ability, and suggested that a similar synergism

may be occurring in human tumors that cooverexpress c-Src and the EGFR or other RTKs. Targets of c-Src action can be inferred from an analysis of its known intracellular substrates, which, besides the cell surface receptors, are almost exclusively proteins that regulate actin cytoskeleton dynamics. Thus, c-Src appears to have the capability of affecting both mitogenic growth pathways and morphogenic pathways that influence cell/matrix and cell/cell interactions, motility, invasiveness, and metastasis. Here the focus is on studies that are beginning to reveal the molecular interactions between c-Src and its substrates (as they relate to malignancy) and the effects phosphorylation by c-Src have on their functions. It is becoming clear that c-Src is an obligate partner in mediating mitogenic signaling of at least two RTKs, specifically the PDGF and EGF receptors, and that in the case of EGFR, c-Src mediates tumorigenic signaling as well. This new information, in turn, can be used to design novel diagnostics and therapeutics to interdict the symbiotic relationship between c-Src and the RTKs.

A number of different growth factor receptors that have been shown to associate with or activate c-Src or Src family members were enumerated in Section I. These included receptors for PDGF, CSF-1, HGF/SF, and EGF, as well as HER2. With the exception of the PDGFR and EGFR, little is known about the role of c-Src in signaling through these receptors, other than the fact that c-Src either associates with the receptor or is activated following specific ligand stimulation. Therefore, we focus our discussion on c-Src interactions with the PDGF and EGF receptors. The data implicating c-Src in PDGF-dependent signaling will be briefly summarized, this being the subject of several other reviews. The bulk of our attention will then be focused on the mechanism of interaction between c-Src and EGFR family members.

1. ROLE FOR c-Src IN SIGNALING FROM THE PDGFR

The first evidence that c-Src participates in PDGFR signaling came from the work of Ralston and Bishop (1985), who first observed that c-Src becomes activated on PDGF stimulation. Kypta *et al.* (1990) later demonstrated that c-Fyn and c-Yes are also activated in a PDGF-dependent manner. Activation of c-Src was shown to be accompanied by a translocation of c-Src from the plasma membrane to the cytosol (Walker *et al.*, 1993), a process that may be linked to internalization of the receptor. PDGF stimulation was also shown to stimulate transient association of Src family members with the PDGFR (Kypta *et al.*, 1990). Association between Src family members and the receptor is believed to involve phosphotyrosine–SH2 interactions, because the SH2 domain of c-Fyn is required for binding to the receptor *in vitro* (Twamley *et al.*, 1992) and mutation to phenylalanine of Y579 and Y581 in the juxtamembrane region of the receptor results in a decrease in both PDGF-induced c-Src activation and binding to the receptor *in*

vivo (Mori *et al.*, 1993). These data and results from *in vitro* peptide binding studies (Alonso *et al.*, 1995) suggest that Y579 and Y581 directly mediate binding of Src family members to the PDGFR.

Interaction of c-Src with the PDGFR appears to have consequences for both c-Src and the PDGFR. Hansen *et al.* (1996) have shown that Y934 in the kinase domain of the PDGFR is phosphorylated by c-Src both *in vitro* and *in vivo*. Expression of a receptor harboring a phenylalanine substitution at residue 934 in intact cells results in a decreased mitogenic signal and an increase in chemotaxis and motility, along with enhanced PLCγ tyrosine phosphorylation. These data suggest that phosphorylation of Y934 by c-Src positively regulates mitogenesis, while negatively regulating cell motility, possibly via a PLCγ-mediated pathway. Activation of c-Src by PDGF is also accompanied by the appearance of novel phosphorylations on c-Src, including two serine phosphorylations, S12 and an unidentified S residue (Gould and Hunter, 1988)), and one tyrosine phosphorylation, Y138 (Broome and Hunter, 1997). Y138 is located in the SH3 domain of c-Src, and phosphorylation of this residue diminishes the ability of peptide ligands to bind the SH3 domain *in vitro*. Mutation of Y138 or Y133 to phenylalanine or complete deletion of the SH3 domain reduces the mitogenic effect of PDGF (Erpel *et al.*, 1996; Broome and Hunter, 1996). The hypothesis that Src family members are required for PDGF-dependent signaling is supported by the inhibitory effects of kinase-inactive c-Src or an antibody specific for the C-terminal domain of Src family members on PDGF-induced BrdU incorporation into newly synthesized DNA (Twamley-Stein *et al.*, 1993).

2. ROLE OF c-Src IN SIGNALING FROM EGFR

In our laboratory, initial attempts to detect EGF-induced alterations in c-Src kinase activity or physical association between c-Src and the EGFR in a panel of nontransformed avian and rodent cell lines were negative, or yielded inconsistent results (Luttrell *et al.*, 1988). Therefore, a direct test of the involvement of c-Src was undertaken, in which wild-type (wt) and mutational variants of c-Src were overexpressed in C3H10T1/2 mouse fibroblasts, and the effect of overexpression of these variants on EGF-induced [^{3}H]thymidine incorporation was examined. Overexpression of wt c-Src resulted in a two- to fivefold increase in [^{3}H]thymidine incorporation above Neo-only controls (Luttrell *et al.*, 1988), whereas overexpression of c-Src harboring inactivating mutations in the kinase, SH2, or myristylation domains resulted not only in a reduction in the enhanced effect of overexpressed wt c-Src but also in a dominant negative effect on endogenous, EGF-induced DNA synthesis (Wilson *et al.*, 1989). These results indicated not only that c-Src is required for mitogenesis stimulated by EGF, but also that c-Src kinase activity, an intact SH2 domain, and membrane association are

necessary to fulfill the role of c-Src in the process. These findings were corroborated by studies in NIH3T3 cells, in which a decrease in EGF-induced BrdU incorporation was observed on microinjection of antibodies to c-Src family members or introduction of a kinase-inactive c-Src cDNA into cells (Roche *et al.*, 1995).

c-Src was also shown to affect EGF-induced tumorigenesis (Maa *et al.*, 1995). In C3H10T1/2 cells, coexpression of c-Src and the HER1 results in synergistic increases in proliferation, colony formation in soft agar, and tumorigenicity in nude mice, as compared to cells overexpressing c-Src or HER1 alone. Furthermore, under conditions of receptor and c-Src overexpression, an EGF-inducible complex between the proteins can be detected. Enhanced tumor growth correlates with the ability of c-Src to associate stably with the receptor, the appearance of two novel tyrosine phosphorylation sites on the receptor, and enhanced phosphorylation of the receptor substrates, Shc and PLCγ. These findings suggest that c-Src association with and phosphorylation of the receptor results in hyperactivation of the receptor and enhanced mitogenic signaling to downstream effectors. Subsequent investigations have revealed that the kinase activity of c-Src is required for the biological synergy between c-Src and overexpressed HER1 (Tice *et al.*, 1998). Kinase-defective c-Src, when expressed in a cell line overexpressing HER1, acts in a dominant negative fashion to inhibit EGF-dependent colony formation in soft agar and tumorigenicity in nude mice. The effects of both wt and kinase-defective c-Src are very striking, with the single wt c-Src or HER1 overexpressors forming barely detectable tumors in nude mice ($<<300$ mm^3) and the c-Src/HER1 double overexpressor forming large tumors (~ 1600 mm^3). In contrast, the HER1/kinase-defective c-Src overexpressors form no palpable tumors. Thus, the extent of tumor inhibition by kinase-defective c-Src is complete in this model system, and the results suggest that the catalytic activity of c-Src may be a fruitful target for human tumor therapy. Interestingly, expression of c-Src variants that bear mutations in either the SH2 or myristylation domains augments, rather than inhibits, tumor formation of cells overexpressing the receptor (D. A. Tice, unpublished). These results are in surprising contrast to those observed when the same c-Src variants are expressed in cells containing normal levels of EGFR (see above and Wilson *et al.*, 1989). The mechanism by which tumor growth is enhanced by these variants is not known.

The mechanism of synergy between wt c-Src and HER1 is beginning to be elucidated. Several groups have now demonstrated an EGF-dependent complex formation between c-Src and the HER1 (Luttrell *et al.*, 1994; Maa *et al.*, 1995; Stover *et al.*, 1995) and an EGF-induced activation of c-Src-specific kinase activity (Osherov and Levitzki, 1994; Oude Weernink *et al.*, 1994). In all instances, these events are seen in cells overexpressing one or both partners, suggesting that the interaction is either transient or low affin-

ity. In addition, there is evidence for phosphorylation of HER1 by c-Src on EGF stimulation. In the C3H10T1/2 murine fibroblasts, two sites of tyrosine phosphorylation on c-Src-associated HER1 have been identified both *in vitro* and *in vivo* as Y845 and Y1101 (Biscardi *et al.,* 1998b). Y845 has also been identified as a c-Src-specific phosphorylation site in A431 cells (Sato *et al.,* 1995) and in MDA-MB-468 breast cancer cells (Biscardi *et al.,* 1998b), whereas two other nonautophosphorylation sites, Y891 and Y920, were identified on the receptor from MCF7 cells (Stover *et al.,* 1995). Tice *et al.* (1998) have shown in 10T1/2 cells that Y845 is the only phosphorylation that is completely dependent on c-Src kinase activity, implicating a direct phosphorylation of the receptor by c-Src at this site, and suggesting that phosphorylation of other c-Src-dependent sites may involve a third component.

Y845 is located in the activation loop of the kinase domain and is highly conserved among all tyrosine kinases, receptor and nonreceptor alike. Its homolog in c-Src is Y416. Phosphorylation at the homologous site in other kinases is required for full enzymatic activation, through ATP and substrate accessibility (Ellis *et al.,* 1986; Fantl *et al.,* 1989; Knighton *et al.,* 1991; van der Geer and Hunter, 1991; Longati *et al.,* 1994; Kato *et al.,* 1994; Russo *et al.,* 1996; Yamaguchi and Hendrickson, 1996; Mohammadi *et al.,* 1996; Hubbard, 1997). In 10T1/2 cells the presence of this phosphorylation on the HER1 correlates with an increase in tyrosine phosphorylation of receptor substrates Shc and PLCγ, and enhanced growth and tumor formation (Maa *et al.,* 1995), consistent with hyperactivation of the receptor. Conversely, the absence of this phosphorylation (in 10T1/2 cells overexpressing receptor and kinase-defective c-Src) correlates with reduced growth and tumor formation. Thus, phosphorylation of Y845 appears to be required for the oncogenic capabilities of the receptor. Interestingly, in all other kinases but HER1, the Y845 homolog is an autophosphorylation site. That Y845 has not been identified as such for the HER1 may be due to the high lability of the phosphorylation (Biscardi *et al.,* 1998b), or to the fact that c-Src appears to be the kinase that phosphorylates it (Tice *et al.,* 1998). Phosphorylation of Y845 also appears to be critical for normal signaling through the receptor. This is supported by recent findings that a Y845F mutation completely ablates EGF or serum-induced DNA synthesis, either in the presence or absence of overexpressed c-Src (Tice *et al.,* 1998). Thus, the ability of c-Src to phosphorylate Y845 is critical for manifestation of both the mitogenic and tumorigenic properties of the receptor.

a. EGF Receptor Internalization

Based on evidence that implicates the actin cytoskeleton as critical for EGFR internalization (Lamaze *et al.,* 1997), and the involvement of c-Src and c-Src substrates in actin dynamics, as well as the localization of c-Src to

membranes of intracellular vesicles (Parsons and Creutz, 1986; Kaplan *et al.*, 1992), it is reasonable to speculate that c-Src enhances EGF-dependent signaling by influencing receptor internalization. One hypothesis is that c-Src enhances mitogenesis and tumorigenesis by inhibiting internalization and prolonging receptor signaling at the plasma membrane. In surprising contrast to this hypothesis, however, it was found in studies of 10T1/2 cells that c-Src overexpression enhances rather than inhibits receptor internalization by increasing steady-state pools of internalized, activated receptors (Ware *et al.*, 1997). Receptor recycling rates are not altered. The kinase activity of c-Src is required for the increase, because overexpression of kinase-deficient c-Src exhibits basal or slightly reduced internalization rates.

How might the increased internalization contribute to the enhanced cell proliferation and tumorigenic potential seen in cells overexpressing wt c-Src? Recent evidence indicates that receptor/SHC/GRB2/SOS complexes are present in endosomes (DiGuglelmo*et al.*, 1994), suggesting that EGF/EGFR complexes continue to signal in the endosomal compartment (Baass *et al.*, 1995; Bevan *et al.*, 1996). Because c-Src overexpression increases the steady-state pool of internalized, activated receptors, c-Src may enhance mitogenic and tumorigenic signaling by promoting the frequency of interactions between receptor complexes in the endosomes and Ras at the plasma membrane.

Although the mechanism by which c-Src affects EGFR internalization is unknown, several possibilities are plausible. First, c-Src may increase the rate of association of the EGFR with components of the endocytic pathway, such as the adaptins (Sorkin and Carpenter, 1993), or Grb2 (Wang and Moran, 1996), which are thought to recruit activated receptors into clathrin-coated pits. Interestingly, Grb2 associates with dynamin (Gout *et al.*, 1993), a GTPase that is critical for the formation and release of the endosome from the plasma membrane. The c-Src SH3 domain is also reported to activate the GTPase activity of dynamin *in vitro* (Herskovits *et al.*, 1993). These considerations suggest that as a second mechanism, overexpression of c-Src could result in the activation or recruitment of a pool of dynamin larger than that in cells expressing normal levels of c-Src.

A third mechanism by which c-Src may affect EGFR internalization is through processes that do not involve clathrin-coated pits, such as through caveolae. Caveolae are small invaginations of the plasma membrane that have been implicated in the transcytosis of macromolecules across capillary endothelial cells, the uptake of small molecules, interactions with actin-based cytoskeleton, and the compartmentalization of certain signaling molecules, including G-protein-coupled receptors, H-Ras and Ras-related GTPases, and members of the Src family of tyrosine kinases (Li *et al.*, 1996a,b). Caveolae are enriched for a specific protein, caveolin, which is a substrate for v-Src (Li *et al.*, 1996a,b), and has also been shown to copurify with c-Src in normal

cells (Lisanti *et al.,* 1994; Henke *et al.,* 1996; Li *et al.,* 1996a,b). Caveolin normally acts as a scaffolding protein to bind inactive signaling molecules, such as Gα subunits, Ras, EGFR, and c-Src (Sargiacomo *et al.,* 1993; Lisanti *et al.,* 1994; Chang *et al.,* 1994; Li *et al.,* 1995; Couet *et al.,* 1997). It has also been shown that caveolin expression is down-modulated in cells transformed by various oncogenes (Koleske *et al.,* 1995), and that reexpression of caveolin in v-*abl*- and H-*ras*-transformed cells will abrogate anchorage-independent growth in these cell lines (Engelman *et al.,* 1997). Caveolin expression has also been shown by differential display and subtractive hybridization techniques to be down-regulated in human mammary carcinomas and several breast tumor cell lines compared with normal breast epithelium (Sager *et al.,* 1994). This evidence suggests that caveolin is inhibitory for transformation and that overexpression of c-Src may be deactivating caveolin through phosphorylation, leading to increased transformation.

b. Evidence for the HER1/c-Src Synergy Model in Human Breast Cancer

Simultaneous overexpression or activation of HER1 and c-Src in a significant portion of human breast tumors suggests that the two molecules might functionally interact in human tumors as they do in the 10T1/2 murine fibroblast model. This question was examined by Biscardi *et al.* (1998a,b), who analyzed a panel of 14 breast tumor cell lines and over 20 tissue samples for levels of HER1 and c-Src overexpression, association between c-Src and HER1, phosphorylation of Y845 and Y1101 on the receptor in complex with c-Src, increases in Shc phosphorylation, MAP kinase activation, and increases in tumor formation in nude mice. A direct correlation was found between the expression levels of c-Src and HER1 and the ability to detect stable interactions between the two kinases, the presence of the novel phosphorylations on the receptor, enhanced phosphorylation of downstream substrates, and tumor formation. Although not direct proof, results from these studies are consistent with those in the 10T1/2 model and suggest that c-Src and HER1 can functionally synergize to promote tumor progression when cooverexpressed in human tumors.

3. c-Src/HER2/*neu* INTERACTIONS

Because HER2/*neu* is so abundantly and frequently overexpressed in human tumors (particularly in breast cancers) and is oncogenic when overexpressed in cultured fibroblasts (DiFiore *et al.,* 1987b), an important question arises as to whether c-Src acts as a cotransducer to tumorigenic signals through HER2 as it does through HER1. Luttrell *et al.* (1994) showed that HER2 can be precipitated by the GSTc–SrcSH2 fusion protein from a hu-

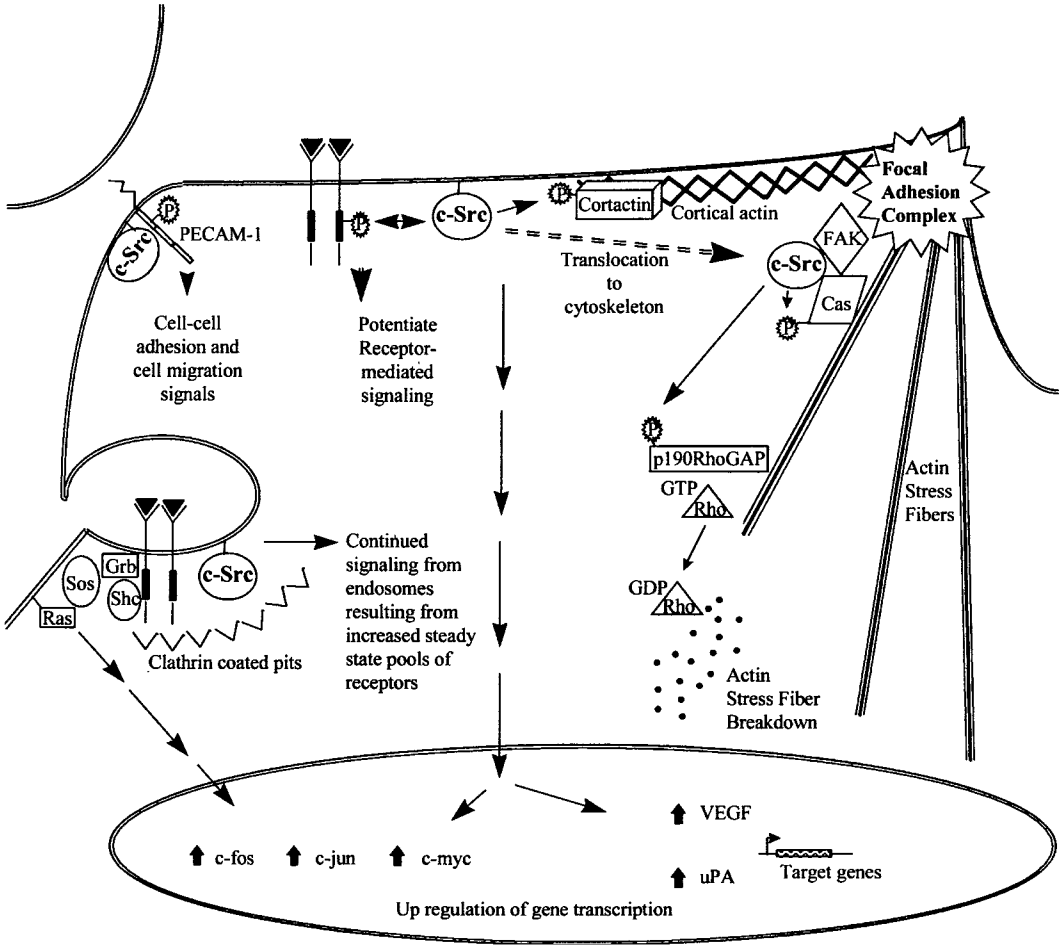
man breast cancer cell extract, suggesting that stable complexes may also form between c-Src and HER2 *in vivo*. c-Src association with and activation by HER2 was also shown in mammary tumors from HER2 transgenic mice (Muthuswamy *et al.*, 1994; Muthuswamy and Muller, 1995). Moreover, in coimmunoprecipitation studies our laboratory has detected c-Src in association with HER2 in 3 of 14 human breast tumor cell lines and in 3 of 13 tumor tissues (Belsches and Parsons, 1998). Cell lines exhibiting this complex respond to HRG mitogenically and tumorigenically, as measured by cell growth assays and colony formation in soft agar, in contrast to those cell lines that express HER2 but form no complex with c-Src. Interestingly, in contrast to the HER1, overexpression of neither HER2 nor c-Src is a prerequisite for detecting association between the two proteins. These data suggest that c-Src may potentiate HER2-dependent tumorigenicity through mechanisms similar as well as dissimilar to those described for HER1.

B. Targets of c-Src

1. TARGETS WHOSE EXPRESSION LEVELS ARE AFFECTED BY c-Src

The preceding discussion provides compelling evidence that RTKs can be direct targets of c-Src. Phosphorylation of specific sites by c-Src appears to regulate the shift from motility to mitogenesis in the case of the PDGFR (Hansen *et al.*, 1996) and the entrance into S phase of the cell cycle in the case of the EGFR (Fig. 3) (Tice *et al.*, 1998). Is c-Src capable of contributing to the malignant phenotype in ways other than through direct regulation of growth factor receptors? One alternative is the ability of c-Src to regulate gene transcription. Barone and Courtneidge (1995) showed that Myc was required to overcome a block of PDGF-induced DNA synthesis by kinase-deficient c-Src, suggesting that Src kinases control the transcriptional activation of Myc, which in turn can induce a program of gene transcription that

Fig. 3 Targets of c-Src and their potential roles in transformation. c-Src associates with and phosphorylates the ligand-activated EGF receptor, thereby potentiating downstream signaling from the receptor. This is manifested by increased levels of phosphorylated receptor substrates and augmented steady-state pools of internalized, activated receptors. In a reciprocal fashion, activated receptors can mediate activation and translocation of c-Src to the cytoskeleton, where it phosphorylates several substrates, including cortactin, p130CAS, and p190RhoGAP. These substrates are central to regulation of actin cytoskeleton rearrangements and thus signals that control morphological transformation and migration. c-Src also contributes to neoplastic development through cell–cell adhesion signaling and up-regulation of gene transcription. Similar types of interactions are thought to occur with other receptor tyrosine kinases known to associate with c-Src.


PECAM-1
c-Src
c-Src
Cortactin
Cortical actin
Focal Adhesion Complex
FAK
c-Src
Cas
Translocation to cytoskeleton
Cell-cell adhesion and cell migration signals
Potentiate Receptor-mediated signaling
p190RhoGAP
GTP
Rho
Actin Stress Fibers
Grb
Sos
Shc
c-Src
Ras
Clathrin coated pits
Continued signaling from endosomes resulting from increased steady state pools of receptors
GDP
Rho
Actin Stress Fiber Breakdown
VEGF
c-fos
c-jun
c-myc
uPA
Target genes
Up regulation of gene transcription

is required for growth. c-Src and v-Src have also been shown to up-regulate transcription of vascular endothelial growth factor (VEGF) (Rak *et al.*, 1995; Mukhopadhyay *et al.*, 1995a,b; Weissenberger *et al.*, 1997). VEGF is a multifunctional cytokine that alters the pattern of gene expression and stimulates the proliferation and migration of endothelial cells that line the walls of microcapillaries. VEGF treatment also renders these same cells hyperpermeable, thereby allowing plasma proteins access to the extracapillary space. This process, in turn, leads to profound alterations in the extracellular matrix that favor angiogenesis (reviewed in Klagsbrun and D'Amore, 1996). Another potent modulator of angiogenesis (reviewed in Tkachuk *et al.*, 1996) and metastasis (reviewed in Andreason *et al.*, 1997) is urokinase-type plasminogen activator (UPA), whose expression is up-regulated by v-Src (Bell *et al.*, 1990, 1993) and whose receptor is found in complex with c-Src family members (Bohuslav *et al.*, 1995). The ability of c-Src to influence gene transcription is a new and emerging question that is receiving considerable attention. However, most of the investigations that focus on the role of c-Src in neoplastic transformation have focused on substrates of c-Src and their contributions to development of the malignant phenotype. c-Src has a number of characterized substrates, most of which have functional connections to the actin cytoskeleton. These different substrates and their potential roles in transformation are discussed below.

2. TARGETS THAT SERVE AS SUBSTRATES OF c-Src

a. Focal Adhesion Kinase

Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that localizes to focal adhesions and contributes to the processes of integrin-mediated cell spreading and migration through regulation of actin cytoskeleton remodeling (reviewed in Parsons and Parsons, 1997). FAK becomes tyrosine phosphorylated in response to various environmental stimuli, such as extracellular matrices and polypeptide and neuropeptide growth factors (reviewed in Schaller and Parsons, 1994). c-Src is intimately involved in FAK-mediated signaling. Activated c-Src (Y527F) is complexed with FAK through binding of the c-Src SH2 domain to Y397, the FAK autophosphorylation site (Schaller *et al.*, 1994; Cobb *et al.*, 1994). In addition, wt c-Src appears to be required for the FAK-mediated, integrin stimulation of mitogen-activated protein kinase (MAPK) (Schlaepfer and Hunter, 1997). Introduction of the amino-terminal half of c-Src, which lacks the kinase domain, reconstitutes integrin-induced MAPK activation in c-Src −/− fibroblasts, thus the process appears to be independent of c-Src kinase activity (Schlaepfer *et al.*, 1997). This finding suggests a potential role for c-Src as a docking protein. The role of c-Src in cell spreading also appears to be independent of the kinase domain, because defects in spreading of fibroblasts derived from c-Src null mice can be restored by the SH2 and SH3 domains but not by the catalytic do-

main of c-Src (Kaplan *et al.*, 1994, 1995). These findings suggest that even though c-Src is activated in response to motogenic factors such as EGF and HGF, it may require only the redistribution of c-Src to focal adhesions to stimulate motility and focal adhesion turnover.

Given that FAK is known to transduce signals involved in the regulation of cell adhesion and motility as well as the anchorage-independent growth of transformed cells, it would not be unexpected to find aberrant expression of FAK in human tumors. Indeed, increased expression or activation of FAK is observed in a number of human tumors, including sarcomas and carcinomas of the breast, prostate, and colon (Weiner *et al.*, 1994; Owens *et al.*, 1995; Withers *et al.*, 1996; Tremblay *et al.*, 1996). As might be expected with involvement of FAK in motility, the highest levels of FAK are seen in metastatic or invasive lesions (Weiner *et al.*, 1994; Owens *et al.*, 1995; Tremblay *et al.*, 1996). An increase in FAK phosphorylation also correlates with increased migration and invasiveness of squamous cell carcinoma cells treated with HGF (Matsumoto *et al.*, 1994). Together, the evidence suggests that signaling through FAK/c-Src complexes in normal and malignant cells is bidirectional. Integrins activate FAK, which can signal through c-Src to activate ERK2, or conversely, engaged growth factor receptors activate c-Src, which can then signal through FAK to mediate motogenic or cytoskeletal responses.

b. p130CAS

p130Cas (CAS) was first identified as a highly tyrosine-phosphorylated protein in cells transformed by a variety of oncogenes (Mayer and Hanafusa, 1990; Auvinen *et al.*, 1995; Salgia *et al.*, 1996) and in normal cells following activation of integrins (Nojima *et al.*, 1995; Petch *et al.*, 1995; Vuori *et al.*, 1996) and stimulation with mitogenic neuropeptides, such as bombesin, vasopressin, and endothelin (Zachary *et al.*, 1992; Seufferlein and Rozengurt, 1994). The role of CAS in integrin or growth factor-mediated signaling is not understood. However, recent evidence indicates that CAS functions like an adapter molecule, binding a number of signaling molecules that participate in cell adhesion, such as FAK (Polte and Hanks, 1995; Harte *et al.*, 1996), PTP-PEST (Garton *et al.*, 1997), and Src family kinases (Polte and Hanks, 1995; Nakamoto *et al.*, 1996). Tyrosine phosphorylation of CAS is increased on adhesion and is largely dependent on c-Src (Vuori *et al.*, 1996; Hamasaki *et al.*, 1996). Thus, the link between cell adhesion, the actin cytoskeleton, and tumorigenesis is repeated, and the common involvement of c-Src in both processes suggests that c-Src may be a critical factor that links them together.

c. Cortactin

The v-Src and c-Src substrate, p75/p80/p85 cortactin, is an actin-binding protein that contains five tandem repeats in the N terminus and an SH3 domain in the extreme C terminus (Wu *et al.*, 1991; Maa *et al.*, 1992; Wu and

Parsons, 1993). The N terminus is responsible for the *in vitro* binding to actin. *In vivo* phosphoamino acid analysis of cortactin from primary chick embryo cells reveals that it contains only serine and threonine phosphorylations, whereas it exhibits constitutive phosphorylation on tyrosine in addition to serine and threonine in cells transformed by activated c-Src (Y527F) (Wu *et al.*, 1991). In immortalized murine 10T1/2 fibroblasts, cortactin has a low basal level of tyrosine phosphorylation that is increased on both EGF stimulation and c-Src overexpression (Wilson and Parsons, 1990; Maa *et al.*, 1992). Cortactin has also been shown to be tyrosine phosphorylated in response to fibroblast growth factors (Zhan *et al.*, 1993, 1994). These observations suggest that cortactin may be a substrate of growth factor receptors as well as of c-Src or that c-Src mediates growth factor-induced tyrosine phosphorylation of cortactin. The findings that cortactin associates with the Src SH2 domain and colocalizes with v-Src in transformed cells (Okamura and Resh, 1995), and that increased tyrosine phosphorylation of cortactin is seen in CSK-deficient cells, favor the notion that c-Src and/or its family members are responsible for phosphorylating cortactin (Nada *et al.*, 1994; Thomas *et al.*, 1995). Interestingly, two phases of EGF-induced cortactin tyrosine phosphorylation can be observed in 10T1/2 cells, one occurring within 2–10 min following stimulation and another occurring later in G_1, with the maximum level seen approximately 9 hr posttreatment. In both cases, the level of phosphorylation is increased by overexpression of c-Src (Maa *et al.*, 1992). These observations raise the question of whether cortactin may function in mid–late G_1 as well as in immediate–early G_1.

Indirect immunofluorescence microscopy of 10T1/2 cells reveals that cortactin is localized within the cytoplasm to punctate sites that are concentrated around the nucleus and colocalized with actin at the plasma membrane and peripheral adhesion site (Maa *et al.*, 1992). This pattern is not altered on EGF treatment or c-Src overexpression in 10T1/2 cells. However, in v-Src- or Y527F–c-Src-transformed cells, cortactin is localized to modified focal adhesions, termed podosomes. The appearance of podosomes is associated with loss of adhesive properties (Marchisio *et al.*, 1987; Wu *et al.*, 1991). Cortactin is also localized to podsome-like cell matrix sites in human tumors that overexpress the protein (including carcinomas of the breast and head and neck) (Schuuring *et al.*, 1992; Brookes *et al.*, 1993; Williams *et al.*, 1993; Schuuring *et al.*, 1993; Meredith *et al.*, 1995; Campbell *et al.*, 1996; van Damme *et al.*, 1997). From these observations, it follows that abnormal subcellular distribution of cortactin in human carcinomas may play a role in deregulating important protein–protein interactions that may be required for the proper formation of cell matrix contact sites. In support of this hypothesis is the correlation of cortactin overexpression with increased invasiveness, metastasis, and a poorer patient prognosis (Williams *et al.*, 1993; Meredith *et al.*, 1995; Takes *et al.*, 1997).

d. p190RhoGAP

p190RhoGAP was first identified as a tyrosine-phosphorylated protein that coprecipitates with p120RasGAP from v-Src-transformed Rat-2 cells (Ellis *et al.,* 1990). p190 has an N-terminal domain that binds GTP (Settleman *et al.,* 1992b; Foster *et al.,* 1994) and a C-terminal GTPase-activating domain (GAP) that is specific for small GTP-binding proteins of the Rho family (Settleman *et al.,* 1992a). p190 is functionally linked to the actin cytoskeleton through its ability to stimulate the conversion of Rho-GTP (which stimulates stress fiber formation) to Rho-GDP (which permits actin disassembly) (Ridley and Hall, 1992). Two phosphorylation sites, Y1087 and Y1105, in the middle portion of the molecule are postulated to mediate binding to the two SH2 domains of p120RasGAP (Bryant *et al.,* 1995; Hu and Settleman, 1997), although of the two sites, only Y1105 phosphorylation can be detected *in vivo.* Overexpression of c-Src in 10T1/2 cells results in an increase in the basal tyrosine phosphorylation of p190, specifically at Y1105 (Roof *et al.,* 1998). This evidence, along with the findings that overexpression of kinase-deficient c-Src decreases the phosphorylation at Y1105 and that c-Src phosphorylates Y1105 *in vitro,* suggests that c-Src is directly responsible for phosphorylation of this residue. Levels of p190 tyrosine phosphorylation are generally correlated with levels of the p190/p120RasGAP complex that can be detected *in vivo,* suggesting that high levels of p190 tyrosine phosphorylation could bind more RasGAP, thereby sequestering RasGAP away from Ras and permitting Ras to remain in the active, GTP-bound state longer. This scenario is consistent with the role of c-Src as a comitogenic signaling partner of growth factor receptors.

Although EGF treatment of 10T1/2 cells does not cause a further increase in tyrosine phosphorylation of p190, it does cause a rapid (seconds to minutes) and transient redistribution of p190 from a diffuse cytoplasmic localization into concentric arcs that radiate away from the nucleus with a time course that mimics EGF-stimulated actin dissolution (Chang *et al.,* 1995). Overexpression of wt c-Src expands the window of time in which EGF-induced actin dissolution and p190 arc formation occur, whereas overexpression of kinase-deficient c-Src contract the window. These results correlate with the level of p190 tyrosine phosphorylation and implicate another role for c-Src in regulating cytoskeletal reorganization, possibly by inactivating Rho through activation and redistribution of p190RhoGAP.

e. Platelet Endothelial Cell Adhesion Molecule

Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a 130-kDa glycoprotein of the immunoglobulin gene superfamily that localizes to points of contact between confluent endothelial cells (Newman *et al.,* 1990; Tanaka *et al.,* 1992). On induction of endothelial sheet migration, PECAM-1 becomes diffusely organized within the cytoplasm, and ectopic expression of

the gene inhibits cell migration, suggesting that translocation from the periphery to the cytoplasm is a mechanism by which the inhibitory action of PECAM-1 is relieved (Schimmenti *et al.*, 1992). The cytoplasmic tail of PECAM-1 is critical for cell surface activity (DeLisser *et al.*, 1994; Yan *et al.*, 1995). It contains immunoreceptor tyrosine-based activation motifs (ITAMS) that are phosphorylated by c-Src *in vitro* and *in vivo* and bind c-Src SH2 domains *in vitro* (Lu *et al.*, 1997). Several lines of evidence suggest that tyrosine phosphorylation is involved in transducing cell migration signals through this molecule (Lu *et al.*, 1996; Pinter *et al.*, 1997). Tyrosines 663 and 686 appear to be the major sites of tyrosine phosphorylation, because mutation of either residue results in a drastic reduction in tyrosine phosphorylation, and mutation of Y686 is associated with a reversal of the PECAM-1-mediated inhibition of cell migration (Lu *et al.*, 1996). Again, phosphorylation by c-Src is a potential mechanism of regulation of a molecule involved in cell–cell contacts and migration, pointing to a role for both PECAM-1 and c-Src in angiogenesis and metastasis.

f. Other Substrates

Additional reports implicate still other c-Src substrates in cell–cell adhesion. Syndecan-1 is a cell surface proteoglycan that interacts with extracellular matrix molecules and growth factors to maintain epithelial cell morphology, anchorage-dependent growth, and inhibition of invasiveness in cell culture assays. The absence of this molecule correlates with a higher grade of transformation and poorer patient prognosis (Inki and Jalkanen, 1996). Its expression is negatively regulated at the level of translation on transformation by polyoma virus middle-T antigen. The effects of middle-T antigen are dependent on association with and activation of c-Src (Levy *et al.*, 1996). c-Src may also function in processes other than those related to cytoskeletal or adhesion dynamics but that still lead or contribute to a transformed phenotype. For example, a role for c-Src in mitosis has been implicated through the identification of a 68-kDa RNA-binding protein, called Sam68 (Src-associated in mitosis), that binds the SH3/2 domains of c-Src (reviewed in Courtneidge and Fumagali, 1994) and is postulated to act through c-Src to regulate microtubule dynamics via association with (Abu-Amer *et al.*, 1997) and phosphorylation of (Matten *et al.*, 1990) tubulin.

V. POTENTIAL THERAPEUTIC APPLICATIONS OF c-Src/HER1 INTERACTIONS

Along with the first evidence for possible roles for receptor and nonreceptor tyrosine kinases in human tumorigenesis has come the development of strategies to inhibit the functions of these classes of enzymes. A plethora of

inhibitors based on various structural and functional characteristics of the enzymes have been developed. First and foremost among these are inhibitors of catalytic activity. Among these inhibitors are the tyrphostins (Burke, 1992; Levitzki and Gazit, 1995), which compete with the protein substrate for access to the catalytic site; genistein (Akiyama *et al.,* 1987), a competitive inhibitor of ATP; lavendustins A and B (Onoda *et al.,* 1989); erbstatin (Imoto *et al.,* 1987); and herbimycin A (Uehara *et al.,* 1986, 1989a,b), which has been shown to promote the ubiquitin-based degradation of the TKs (Sepp-Lorenzino *et al.,* 1995). Other inhibitors are designed to prevent interactions mediated through SH2 and SH3 domains (peptidomimetics) (Smithgall, 1995; Plummer *et al.,* 1996, 1997) or to prevent myristylation (*N*-fatty acyl glycinal compounds) (Shoji *et al.,* 1990). In numerous cases these reagents have been demonstrated to be antiproliferative (Clark *et al.,* 1996; Traxler *et al.,* 1997; Hartmann *et al.,* 1997). In other instances membrane penetrance of the drug has been a problem in testing their efficacies in tissue culture and animal models (Gilmer *et al.,* 1994).

Studies characterizing the molecular interactions between c-Src and HER1 have revealed an additional target for drug design, specifically the sequences surrounding Y845 of HER1. Phosphorylation of this site by c-Src appears to be required for the mitogenic and tumorigenic aspect of receptor function, as shown by the inability of kinase-defective c-Src to phosphorylate Y845 and the nonfunctionality of the mutant Y845F receptor. In human tumors that overexpress c-Src and HER1, inhibiting the ability of c-Src to phosphorylate Y845 might reduce the tumorigenic potential of the overexpressed receptor as well as the ability of c-Src to synergize with the receptor. Such inhibition might be accomplished by a Y845 peptidomimetic. The advantages appear to be that this inhibition targets an enzyme/substrate interaction that occurs to the greatest degree in those cells that overexpress both players (c-Src and HER1, respectively), namely, cancer cells. In no normal cells are these two molecules known to be simultaneously overexpressed. For example, in the adult, the highest levels of c-Src are found in platelets (Golden *et al.,* 1986) and in cells of the nervous system, whereas high levels of HER1 are found in the liver and kidney (Nexo and Kryger-Baggesen, 1989). In theory, therefore, the Y845 peptidomimetic might be more likely to target the tumor cells than the normal cells, thus providing a potential "tumor-specific" drug for cancers such as carcinomas of the colon, breast, and lung.

REFERENCES

Abu-Amer, Y., Ross, F. P., Schlesinger, P., Tondravi, M. M., and Teitelbaum, S. L. (1997). *J. Cell Biol.* **137**, 247–258.

Adnane, J., Gaudray, P., Simon, M.-P., Simony-Lafontaine, J., Jeanteur, P., and Theillet, C. (1989). *Oncogene* **4**, 1389–1395.

Adnane, J., Gaudray, P., Dionne, C., Crumley, G., Jaye, M., Schlessinger, J., Jeanteur, P., Birnbaum, D., and Theillet, C. (1991). *Oncogene* **6**, 659–663.
Agthoven, T. V., Agthoven, T. L., Portengen, H., Foekens, J. A., and Dorssers L. C. (1992). *Cancer Res.* **52**, 5082–5088.
Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M., and Fukami, Y. (1987). *J. Biol. Chem.* **262**, 5592–5595.
Alexander, R. J., Panja, A., Kaplan-Liss, E., Mayer, L., and Raicht, R. F. (1996). *Dig. Dis. Sci.* **41**, 660–669.
Alonso, G., Koegl, M., Mazurenko, N., and Courtneidge, S. A. (1995). *J. Biol. Chem.* **270**, 9840–9848.
Amini, S., DeSeau, V., Reddy, S., Shalloway, D., and Bolen, J. B. (1986a). *J. Virol.* **6**, 2305–2316.
Amini, S., Lewis, J. R., Israel, M. A., Butel, J. S., and Bolen, J. B. (1986b). *J. Virol.* **57**, 357–361.
Andreason, P. A., Kjoller, L., Christensen, L., and Duffy, M. J. (1997). *Int. J. Cancer* **72**, 1–22.
Arnholdt, H., Diebold, J., Kuhlmann, B., and Lohrs, U. (1991). *Cell Pathol.* **61**, 75–80.
Arnold, S. F., and Notides, A. C. (1995). *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7475–7479.
Arnold, S. F., Obourn, J. D., Jaffe, H., and Notides, A. C. (1995a). *Mol. Endocrinol.* **9**, 24–33.
Arnold, S. F., Vorojeikina, D. P., and Notides, A. C. (1995b). *J. Biol. Chem.* **270**, 30205–30212.
Arnold, S. F., Melamed, M., Vorojeikina, D. P., Notides, A. C., and Sasson, S. (1997). *Mol. Endocrinol.* **11**, 48–53.
Asimakopolous, F. A., White, N. J., Nacheva, E., and Green, A. R. (1994). *Blood* **84**, 3086–3094.
Auger, K. R., Serunian, L. A., Soltoff, S. P., Libby, P., and Cantley, L. C. (1989). *Cell* **57**, 167–175.
Auricchio, F., Migliaccio, A., Di Domenico, M., and Nola, E. (1987). *EMBO J.* **6**, 2923–2929.
Auvinen, M. A., Paasinen-Sohns, A., Hirai, H., Andersson, L. C., and Holtta, E. (1995). *J. Virol.* **15**, 6513–6525.
Baass, P. C., Di Guglielmo, G. M., Authier, F., Posner, B. I., and Bergeron, J. J. (1995). *Trends Endocrinol. Metabol.* **5**, 465–470.
Bacus, S. S., Chin, D., Yarden, Y., Zelnick, C. R., and Stern, D. F. (1996). *Am. J. Pathol.* **148**, 549–558.
Baiocchi, G., Kavanagh, J., Talpaz, M. Wharton, J., Gutterman, J., and Kurzrock, R. (1991). *Cancer* **67**, 990–996.
Barker, K. T., Jackson, L. E., and Crompton, M. R. (1997). *Oncogene* **15**, 799–805.
Barnekow, A., and Gessler, M. (1986). *EMBO J.* **5**, 701–705.
Barnes, D. M., Bartkova, J., Camplejohn, R. S., Gullick, W. J., Smith, P. J., and Millis, R. R. (1992). *Eur. J. Cancer* **28**, 644–648.
Barnhill, R. L., Xiao, M., Graves, D., and Antoniades, H. N. (1996). *Br. J. Dermatol.* **135**, 898–904.
Barone, M. V., and Courtneidge, S. A. (1995). *Nature (London)* **378**, 509–512.
Battaglia, F., Scambia, G., Rossi, G., Benedetti, P., Bellatone, R., Pollizi, G., Querzoli, P., Negrinie, R., Jacobelli, S., Crucittie, F., and Mancuso, S. (1988). *Eur. J. Cancer Clin. Oncol.* **24**, 1685–1690.
Beato, M., Herrlich, P., and Schutz, G. (1995). *Cell* **83**, 851–857.
Bell, S. M., Brackenbury, R. W., Leslie, N. D., and Degen, J. L. (1990). *J. Biol. Chem.* **265**, 1333–1338.
Bell, S. M., Connolly, D. C., Maihle, N. J., and Degen, J. L. (1993). *Mol. Cell. Biol.* **13**, 5888–5897.
Bellusci, S., Moens, G., Gaudino, G., Comoglio, P., Nakamura, T., Thiery, J. P., and Jouanneau, J. (1994). *Oncogene* **9**, 1091–1099.

Belsches, A., and Parsons, S. (1989). In preparation.
Berchuck, A., and Boyd, J. (1995). *Cancer Suppl.* **76**, 2034–2040.
Berlanga, J., Vara, J., Martin-Perez, J., and Garcia-Ruiz, J. (1995). *Mol. Endocrinol.* **9**, 1461–1467.
Betsholtz, C. (1995). *Int. J. Dev. Biol.* **39**, 817–825.
Bevan, A. P., Drake, P. G., Bergeron, J. J., and Posner, B. I. (1996). *Trends Endocrinol.* **7**, 13–21.
Biscardi, J. S., Belsches, A. P., and Parsons, S. J. (1998a). *Mol. Carcinogen.* **21**, 261–272.
Biscardi, J. S., Maa, M. C., Cox, M. E., Leu, T. H., and Parsons, S. J. (1998b). Submitted.
Bishop, J. M. (1983). *Annu. Rev. Biochem.* **52**, 301–354.
Bjelfman, C., Hedborg, F., Johansson, I., Nordenskjold, M., and Pahlman, S. (1990). *Cancer Res.* **50**, 6908–6914.
Boccaccio, C., Ando, M., Tamagnone, L., Bardelli, A., Michiell, P., Battistini, C., and Comoglio, P. M. (1998). *Nature (London)* **391**, 285–288.
Bohuslav, J., Horejsi, V., Hansmann, C., Stockl, J., Weidle, U. H., Majdie, O., Bartke, I., Knapp, W., and Stockinger, H. (1995). *J. Exp. Med.* **181**, 1381–1390.
Bolen, J. B., Veillette, A., Schwartz, A. M., DeSeau, V., and Rosen, N. (1987a). *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2251–2255.
Bolen, J. B., Veillette, A., Schwartz, A. M., DeSeau, V., and Rosen, N. (1987b). *Oncogene Res.* **1**, 149–168.
Bolla, M., Chedin, M., Souvignet, C., Marron, J., Arnould, C., and Chambaz, E. (1990). *Breast Cancer Res. Treat.* **16**, 97–102.
Bonfini, L., Migliaccio, E., Pelicci, G., Lanfrancone, L., and Pelicci, P. G. (1996). *Trends Cell Biol.* **21**, 257–261.
Borg, A., Tandon, A. K., Sigurdsson, H., Clark, G. M., Ferno, M., Fuqua, S. A., Killander, D., and McGuire, W. L. (1990). *Cancer Res.* **50**, 4332–4337.
Bornfeldt, K. E., Raines, E. W., Graves, L. M., Skinner, M. P., Krebs, E. G., and Ross, R. (1995). *Ann. N.Y. Acad. Sci.* **766**, 416–430.
Bottaro, D. P., Rubin, J. S., Faletto, D. L., Chan, A. M., Kmiecik, T. E., Vande Woude, G. F., and Aaronson, S. A. (1991). *Science* **251**, 801–804.
Bourette, R. P., Myles, G. M., Choi, J. L., and Rohrschneider, L. R. (1997). *EMBO J.* **19**, 5880–5893.
Brickell, P. M. (1992). *Crit. Rev. Oncogen.* **3**, 401–446.
Brizuela, L., Ulug, E. T., Jones, M. A., and Courtneidge, S. A. (1995). *Eur. J. Immunol.* **25**, 385–393.
Brookes, S., Lammie, G. A., Schuuring, E., de Boer, C., Michalides, R., Dickson, C., and Peters, G. (1993). *Genes Chromosom. Cancer* **6**, 222–231.
Broome, M. A., and Hunter, T. (1996). *J. Biol. Chem.* **271**, 16798–16806.
Broome, M. A., and Hunter, T. (1997). *Oncogene* **14**, 17–34.
Brown, M. T., and Cooper, J. A., (1996). *Biochim. Biophys. Acta* **1287**, 121–149.
Bruckner, A., Filderman, A. E., Kirchheimer, J. C., Binder, B. R., and Remold, H. G. (1992). *Cancer Res.* **52**, 3043–3047.
Brunton, V. G., Ozanne, B. W., Paraskeva, C., and Frame, M. C. (1997). *Oncogene* **14**, 283–293.
Bryant, S. S., Briggs, S., Smithgall, T. E., Martin, G. A., McCormick, F., Chang, J. H., Parsons, S. J., and Jove, R. (1995). *J. Biol. Chem.* **270**, 17947–17952.
Bu, R., Purushotham, K. R., Kerr, M., Tao, Z., Jonsson, R., Olofsson, J., and Humphreys-Beher, M. G. (1996). *Proc. Soc. Exp. Biol. Med.* **211**, 257–264.
Budde, R. J., Ke, S., and Levin, V. A. (1994). *Cancer Biochem. Biophys.* **14**, 171–175.
Bunone, G., Briand, P. A., Miksicek, R. J., and Picard, D. (1996). *EMBO J.* **15**, 2174–2183.
Burgess, W. H., and Maciag, T. (1989). *Annu. Rev. Biochem.* **58**, 575–606.
Burke, T. R. (1992). *Biorg. Med. Chem. Lett.* **12**, 1711.
Burthem, J., Baker, P. K., Hunt, J. A., and Cawley, J. C. (1994). *Blood* **83**, 1381–1389.

Campbell, D. H., deFazio, A., Sutherland, R. L., and Daly, R. J. (1996). *Int. J. Cancer* **68**, 485–492.
Cance, W. G., Craven, R. J., Bergman, M., Xu, L., Alitalo, K., and Liu, E. T. (1994). *Cell Growth Differ.* **5**, 1347–1355.
Cartwright, C. A., Eckhart, W., Simon, S., and Kaplan, P. L. (1987). *Cell* **49**, 83–91.
Cartwright, C. A., Kamps, M. P., Meisler, A. I., Pipas, J. M., and Eckhart, W. (1989). *J. Clin. Invest.* **83**, 2025–2033.
Cartwright, C. A., Meisler, A. I., and Eckhart, W. (1990). *Proc. Natl. Acad. Sci. U.S.A.* **87**, 558–562.
Cartwright, C. A., Coad, C. A., and Egbert, B. M. (1994). *J. Clin. Invest.* **93**, 509–515.
Castoria, G., Migliaccio, A., Bilancio, A., Pagano, M., Abbondanza, C., and Auricchio, F. (1996). *Int. J. Cancer* **65**, 574–583.
Chaffanet, M., Chauvin, C., Laine, M., Berger, F., Chedin, M., Rost, N., Nissou, M. F., and Benabid, A. L. (1992). *Eur. J. Cancer* **28**, 11–17.
Chang, W. J., Ying, Y. S., Rothberg, K. G., Hooper, N. M., Turner, A. J., Gambliel, H. A., De Gunzburg, J., Mumby, S. M., Gilman, A. G., and Anderson, R. G. (1994). *J. Cell Biol.* **126**, 127–138.
Chang, J. H., Gill, S., Settleman, J., and Parsons, S. J. (1995). *J. Cell Biol.* **130**, 1–14.
Chrysogelos, S. A., and Dickson, R. B. (1994). *Breast Cancer Res. Treat.* **29**, 29–40.
Claesson-Welsh, L., and Heldin, C. H. (1989). *Acta Oncol.* **28**, 331–334.
Claesson-Welsh, L., Hammacher, A., Westermark, B., Heldin, C. H., and Nister, M. (1989). *J. Biol. Chem.* **264**, 1742–1747.
Clark, J. W., Santos-Moore, A., Stevenson, L. E., and Frackelton, J. (1996). *Int. J. Cancer* **65**, 186–191.
Clarke, R., Brunner, N., Katz, D., Glanz, P., Dickson, R. B., Lippman, M. E., and Kern, F. G. (1989). *Mol. Endocrinol.* **3**, 372–380.
Cobb, B. S., Schaller, M. D., Leu, T. H., and Parsons, J. T. (1994). *J. Virol.* **14**, 147–155.
Coltrera, M. D., Wang, J., Porter, P. L., and Gown, A. M. (1995). *Cancer Res.* **55**, 2703–2708.
Cook, R. M., Miller, Y. E., and Bunn, J. R. (1993). *Curr. Prob. Cancer* **17**, 69–141.
Cooper, C. S., Park, M., Blair, D. G., Tainsky, M. A., Huebner, K., Croce, C. M., and Vande Woude, G. F. (1984). *Nature* **311**, 29–33.
Couet, J., Sargiacomo, M., and Lisanti, M. P. (1997). *J. Biol. Chem.* **272**, 30429–30438.
Coughlin, S. R., Escobedo, J. A., and Williams, L. T. (1989). *Science* **243**, 1191–1194.
Courtneidge, S. A., and Fumagali, S. (1994). *Trends Cell Biol.* **4**, 345–347.
Courtneidge, S. A., Dhand, R., Pilat, D., Twamley, G. M., Waterfield, M. D., and Roussel, M. (1993). *EMBO J.* **12**, 943–950.
Dardik, A., and Schultz, R. M. (1991). *Development* **113**, 919–930.
Davidson, N. E., Gelmann, E. P., Lippman, M. E., and Dickson, R. B. (1987). *Mol. Endocrinol.* **1**, 216–223.
Delarue, J. C., Friedman, S., Mouriesse, H., May-Levin, F., Sancho-Garnier, H., and Contesso, G. (1988). *Breast Cancer Res. Treat.* **11**, 173–178.
DeLisser, H. M., Chilkotowsky, J., Yan, H. C., Daise, M. L., Buck, C. A., and Albelda, S. M. (1994). *J. Cell Biol.* **124**, 195–203.
Denhardt, D. T. (1996). *Biochem. J.* **318**, 729–747.
De Potter, C. R., Beghin, C., Makar, A. P., Vandekerckhove, D., and Roels, H. J. (1990). *Int. J. Cancer* **45**, 55–58.
DeSeau, V., Rosen, N., and Bolen, J. B. (1987). *J. Cell. Biochem.* **35**, 113–128.
Di Domenico, M., Castoria, G., Biolancio, A., Migliaccio, A., and Auricchio, F. (1996). *Cancer Res.* **56**, 4516–4521.
DiFiore, P. P., Pierce, J. H., Fleming, T. P. Hazan, R., Ullrich, A., King, C. R., Schlessinger, J., and Aaronson, S. A. (1987a). *Cell* **51**, 1063–1070.

DiFiore, P. P., Pierce, J. H., Kraus, M. H., Segatto, O., King, C. R., and Aaronson, S. A. (1987b). *Science* **237**, 178–182.

DiGuglelmo, G. M., Baass, P. C., Ou, W. J., Posner, B. I., and Bergeron, J. J. (1994). *EMBO J.* **13**, 4269–4277.

Di Marco, E., Pierce, J. H., Fleming, T. P., Kraus, M. H., Molloy, C. J., Aaronson, S. A., and Di Fiore, P. P. (1989). *Oncogene* **4**, 831–838.

Di Renzo, M. F., Narsimhan, R. P., Olivero, M., Bretti, S., Giordano, S., Medico, E., Gaglia, P., Zara, F., and Comoglio, P. M. (1991). *Oncogene* **6**, 1997–2003.

Di Renzo, M. F., Olivero, M., Ferro, S., Prat, M., Bongarzone, I., Pilotti, S., Belfiore, A., Costantino, A., Vigneri, R., Pierotti, M. A. *et al.* (1992). *Oncogene* **7**, 2549–2553.

Dittadi, R., Donisi, P. M., Brazzale, A., Cappellozza, L., Bruscagnin, G., and Gion, M. (1993). *Br. J. Cancer* **67**, 7–9.

Durst, M., Croce, C. M., Gissmann, L., Schwarz, E., and Huebner, K. (1987). *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1070–1074.

Ebert, M., Yokoyama, M., Friess, H., Kobrin, M. S., Buchler, M. W., and Korc, M. (1995). *Int. J. Cancer* **62**, 529–535.

Eck, M. J., Atwell, S. K., Shoelson, S. E., and Harrison, S. C. (1994). *Nature (London)* **368**, 764.

Egan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Sizeland, A. M., and Weinberg, R. A. (1993). *Nature (London)* **363**, 45–51

Ekstrand, A. J., Sugawa, N., James, C. D., and Collins, V. P. (1992). *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4309–4313.

Ekstrand, A. J., Longo, N., Hamid, M. L., Olson, J. J., Liu, L., Collins, V. P., and James, C. D. (1994). *Oncogene* **9**, 2313–2320.

Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A., and Rutter, W. J. (1986). *Cell* **45**, 721–732.

Ellis, C., Moran, M., McCormick, F., and Pawson T. (1990). *Nature (London)* **343**, 377–380.

Ely, C. M., Tomiak, W. M., Allen, C. M., Thomas, L., Thomas, G., and Parsons, S. J. (1994). *J. Neurochem.* **62**, 923–933.

Empereur, S., Djelloul, S., Di Gioia, Y., Bruyneel, E., Marrel, M., Van Hengel, J., Van Roy, F., Comoglio, P., Courtneidge, S., Paraskeva, C., Chastre, E., and Gespach, C. (1997). *Br. J. Cancer* **75**, 241–250.

Engelman, J. A., Wykoff, C. C., Yasuhara, S., Song, K. S., Okamoto, T., and Lisanti, M. P. (1997). *J. Biol. Chem.* **272**, 16374–16381.

Erpel, T., and Courtneidge, S. A. (1995). *Curr. Opin. Cell Biol.* **7**, 176–182.

Erpel, T., Alonso, G., Roche, S., and Courtneidge, S. A. (1996). *J. Biol. Chem.* **271**, 16807–16812.

Faksvag, H., Akslen, L. A., Varhaug, J. E., and Lillehaug, J. R. (1996). *Cancer Res.* **56**, 1184–1188.

Fantl, W. J., Escobedo, J. A., and Williams, L. T. (1989). *Mol. Cell. Biol.* **9**, 4473–4478.

Favot, P., Yue, X., and Hume, D. A. (1995). *Oncogene* **11**, 1371–1381.

Fazioli, F., Bottaro, D. P., Minichiello, L., Auricchio A., Wong, W. T., Segatto, O., and Di Fiore, P. P. (1992). *J. Biol. Chem.* **267**, 5155–5161.

Fazioli, F., Minichiello, L., Matoskova, V., Castagnino, P., Miki, T., Wong, W. T., and Di Fiore, P. P. (1993a). *EMBO J.* **12**, 3799–3808.

Fazioli, F., Minichiello, L., Matoskova, B., Wong, W. T., and Di Fiore, P. P. (1993b). *Mol. Cell. Biol.* **13**, 5814–5828.

Filderman, A. E., Bruckner, A., Kacinski, B. M., Deng, N., and Remold, H. G. (1992). *Cancer Res.* **52**, 3661–3666.

Fischer-Colbrie, J., Witt, A., Heinzl, H., Speiser, P., Czerwenka, K., Sevelda, P., and Zeillinger, R. (1997). *Anticancer Res.* **17**, 613–620.

Fitzer-Attas, C. J., Do, M. S., Feigelson, S., Vadai, E., Feldman, M., and Eisenbach, L. (1997). *Oncogene* **15**, 1545–1554.
Fitzpatrick, S. L., Brightwell, J., Wittliff, J., Barrows, G. H., and Schultz, G. S. (1984). *Cancer Res.* **44**, 3448–3453.
Folkman, J., Klagsbrun, M., Sasse, J., Wadzinski, M., Ingber, D., and Vlodavsky, I. (1988). *Am. J. Pathol.* **130**, 393–400.
Foster, R., Hu, K. Q., Shaywitz, D. A., and Settleman, J. (1994). *J. Virol.* **14**, 7173–7181.
Garcia, R., Parikh, N. U., Saya, H., and Gallick, G. E. (1991). *Oncogene* **6**, 1983–1989.
Garcia, d. P., Adams, G. P., Sundareshan, P., Wong, A. J., Testa, J. R., Bigner, D. D., and Weiner, L. M. (1993). *Cancer Res.* **53**, 3217–3220.
Garton, A. J., Burnham, M. R., Bouton, A. H., and Tonks, N. K. (1997). *Oncogene* **15**, 877–885.
Gassmann, M., Casagranda, F., Orioll, D., Simon, H., Lal, C., Klein, R., and Lemke, G. (1995). *Nature (London)* **378**, 390–394.
Gee, C. E., Griffin, J., Sastre, L., Miller, L. J., Springer, T. A., Piwnica-Worms, H., and Roberts, T. M. (1986). *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5131–5135.
Gilmer, T., Rodriguez, M., Jordan, S., Crosby, R., Alligood, K., Green, M., Kimery, M., Wagner, C., Kinder, D., Charifson, P., *et al.* (1994). *J. Biol. Chem.* **269**, 31711–31719.
Giordano, S., Bardelli, A., Zhen, Z., Mendard, S., Ponzetto, C., and Comoglio, P. M. (1997). *Proc. Natl. Acad. Sci. U.S.A.* **94**, 13868–13872.
Golden, A., Nemeth, S. P., and Brugge, J. S. (1986). *Proc. Natl. Acad. Sci. U.S.A.* **83**, 852–856.
Gonzatti-Haces, M., Seth, A., Park, M., Copeland, T., Oroszlan, S., and Vande Woude, G. F. (1988). *Proc. Natl. Acad. Sci. U.S.A.* **85**, 21–25.
Gould, K. L., and Hunter, T. (1988). *J. Virol.* **8**, 3345–3356.
Gout, I., Dhand, R., Hiles, I. D., Fry, M. J., Panayotou, G., Das, P., Truong, O., Totty, N. F., Hsuan, J., Booker, G. W., and Campbell, I. A., and Waterfield, M. D. (1993). *Cell* **75**, 25–36.
Grano, M., Galimi, F., Zambonin, G., Colucci, S., Cottone, E., Zamibonin, Z., and Comoglio, P. M. (1996). *Proc. Natl. Acad. Sci. U.S.A.* **93**, 7644–7648.
Grunt, T. W., Saceda, M., Martin, M. B., Lupu, R., Dittrich, E., Krupitza, G., Harant, H., Huber, H., and Dittrich, C. (1995). *Int. J. Cancer* **63**, 560–567.
Gusterson, B. A., Gelber, R. D., Goldhirsch, A., Price, K. N., Save-Soderborgh, J., Anbazhagan, R., Styles, J., Rudenstam, C. M., Golouh, R., and Reed, R. (1992). *J. Clin Oncol.* **10**, 1049–1056.
Guy, C. T., Muthuswamy, S. K., Cardiff, R. D., Soriano, P., and Muller, W. J. (1994). *Genes Dev.* **8**, 23–32.
Gyetko, M., Chen, G., McDonald, R., Goodman, R., Huffnagle, G., Wilkerson, C., Fuller, J., and Toews, G. (1996). *J. Clin. Invest.* **97**, 1818–1826.
Halaban, R., Rubin, J., Funasaka, Y., Cobb, M., Boulton, T., Faletto, D., Rosen, E., Chan, A., Yoko, K., White, W., Cook, C., and Moellmann, G. (1992). *Oncogene* **7**, 2195–2206.
Hamasaki, K., Mimura, T., Morino, N., Furuya, H., Nakamoto, T., Aizawa, S., Morimoto, C., Yazaki, Y., Hirai, H., and Nojima, Y. (1996). *Biochem. Biophys. Res. Commun.* **222**, 338–343.
Hanks, S. J., Quinn, A. M., and Hunter, T. (1988). *Science* **241**, 42–52.
Hannig, G., Ottilie, S., and Schartl, M. (1991). *Oncogene* **6**, 361–369.
Hansen, K., Johnell, M., Siegbahn, A., Rorsman, C., Engstrom, U., Wernstedt, C., Heldin, C. H., and Ronnstrand, L. (1996). *EMBO J.* **15**, 5299–5313.
Hansen, L. A., Alexander, N., Hogan, M. E., Sundberg, J. P., Dlugosz, A., Threadgill, D. W., Magnuson, T., and Yuspa, S. H. (1997). *Am. J. Pathol.* **150**, 1959–1975.
Harris, J. R., Lippmann, M. E., Veronesi, U., and Willett, W. (1992). *N. Engl. J. Med.* **327**, 473–480.
Harte, M. T., Hildebrand, J. D., Burnham, M. R., Bouton, A. H., and Parsons, J. T. (1996). *J. Biol. Chem.* **271**, 13649–13655.

Hartmann, F., Horak, E. M., Cho, C., Lupu, R., Bolen, J. B., Stetler-Stevenson, M. A., Pfreundschuh, M., Waldmann, T. A., and Horak, I. D. (1997). *Int. J. Cancer* **70,** 221–229.
Heldin, C. H. (1996). *Cancer Surv.* **27,** 7–24.
Heldin, C. H., and Westermark, B. (1990). *Cell Reg.* **1,** 555–566.
Henke, R. C., Hancox, K. A., and Jeffrey, P. L. (1996). *J. Neurosci. Res.* **45,** 617–630.
Hennipman, A., van Oirschot, B. A., Smits, J., Rijksen, G., and Staal, G. E. (1989). *Cancer Res.* **49,** 516–521.
Herskovits, J. S., Shpetner, H. S., Burgess, C. C., and Vallee, R. B. (1993). *Proc. Natl Acad. Sci. U.S.A.* **90,** 11468–11472.
Hollings, P. E. (1994). *Genes Chromosom. Cancer* **11,** 21–28.
Holmes, T. C., Fadool, D. A., Ren, R., and Levitan, I. B. (1996). *Science* **274,** 2089–2091.
Hu, K. Q., and Settleman, J. (1997). *EMBO J.* **16,** 473–483.
Hubbard, S. R. (1997). *EMBO J.* **16,** 5572–5581.
Hudziak, R. M., Schlessinger, J., and Ullrich, A. (1987). *Proc. Natl. Acad. Sci. U.S.A.* **84,** 7159–7163.
Huff, J. L., Jelinek, M. A., Borgman, C. A., Lansing, T. J., and Parsons, J. T. (1993). *Proc. Natl. Acad. Sci. U.S.A.* **90,** 6140–6144.
Hume, D. A., Yue, X., Ross, I. L., Favot, P., Lichanska, A., and Ostrowski, M. C. (1997). *Mol. Reprod. Dev.* **46,** 46–53.
Humphrey, P. A., Wong, A. J., Vogelstein, B., Zalutsky, M. R., Fuller, G. N., Archer, G., Friedman, H. S., Kwatra, M. M., Bigner, S. H., and Bigner, D. D. (1990). *Proc. Natl. Acad. Sci. U.S.A.* **87,** 4207–4211.
Hynes, N. E., and Stern, D. F. (1994). *Biochim. Biophys. Acta* **1198,** 165–184.
Hynes, N. E., Gerber, H. A., Saurer, S., and Groner, B. (1989). *J. Cell Biochem.* **39,** 167–173.
Ignar-Trowbridge, D. M., Nelson, K. G., Bidwell, M. C., Curtis, S. W., Washburn, T. G., McLachlan, J. A., and Korach, K. S. (1992). *Proc. Natl. Acad. Sci. U.S.A.* **89,** 4658–4662.
Ignar-Trowbridge, D. M., Teng, C. T., Ross, K. A., Parker, M. G., Korach, K. S., and McLachlan, J. A. (1993). *Mol. Endocrinol.* **7,** 992–998.
Imoto, M., Umezawa, K., Sawa, T., Takeuchi, T., and Umezawa, H. (1987). *Biochem. Int.* **15,** 989–995.
Inki, P., and Jalkanen, M. (1996). *Ann. Med.* **28,** 63–67.
Insogna, K., Tanaka, S., Neff, L., Horne, W., Levy, J., and Baron, R. (1997). *Mol. Reprod. Dev.* **46,** 104–108.
Jacobs, C., and Rubsamen, H. (1983). *Cancer Res.* **43,** 1696–1702.
Jankowski, J., Coghill, G., Hopwood, D., and Wormsley, K. G. (1992). *Gut* **33,** 1033–1038.
Jeffers, M., Rong, S., Anver, M., and Vande Woude, G. F. (1996a). *Oncogene* **13,** 853–861.
Jeffers, M., Rong, S., and Vande Woude, G. F. (1996b). *Mol. Cell. Biol.* **16,** 1115–1125.
Jeffers, M., Schmidt, L., Nakaigawa, N., Webb, C. P., Weirich, G., Kishida, T., Zbar, B., and Vande Woude, G. F. (1997). *Proc. Natl. Acad. Sci. U.S.A.* **94,**11445–11450.
Johnson, D., and Williams, L. T. (1993). *J. Neurooncol.* **18,** 207–216.
Johnston, C., Cox, H., Gomm, J., and Coombes, R. (1995). *Biochem. J.* **306,** 609–616.
Joseph, A., Weiss, G. H., Jin, L., Fuchs, A., Chowdhury, S., O'Shaughnessy, P., Goldberg, I. D., and Rosen, E. M. (1995). *J. Natl. Cancer Inst.* **87,** 373–377.
Juang, S. H., Carvajal, M. E., Whitney, M., Liu, Y., and Carraway, C. A. (1996). *Oncogene* **12,** 1033–1042.
Kacinski, B. M., Carter, D., Mittal, K., Yee, L. D., Scata, K. A., Donofrio, L., Chambers, S. K., Wang, K. I., Yangfeng, T., Rohrschneider, L. R., and Rothwell, V. M. (1990). *Am. J. Pathol.* **137,** 135–147.
Kamalati, T., Jolin, H. E., Mitchell, P. J., Barker, K. T., Jackson, L. E., Dean, C. J., Page, M. J., Gusterson, B. A., and Crompton, M. R. (1996). *J. Biol. Chem.* **271,** 30956–30963.
Kameda, T., Yasui, W., Yoshida, K., Tsujino, T., Nakayama, H., Ito, M., Ito, H., and Tahara, E. (1990). *Cancer Res.* **50,** 8002–8009.

Kan, M., Zhang, G. H., Zarnegar, R., Michalpoulos, G., Myoken, Y., McKeehan, W. L., and Stevens, J. L. (1991). *Biochem. Biophys. Res. Commun.* **174,** 331–337.
Kanai, M., Goke, M., Tsunekawa, S., and Podolsky, D. (1997). *J. Biol. Chem.* **272,** 6621–6628.
Kanda, H., Tajima, H., Lee, G. H., Nomura, K., Ohtake, K., Matsumoto, K., Nakamura, T., and Kitagawa, T. (1993). *Oncogene* **8,** 3047–3053.
Kaplan, D. R., Morrison, D. K. Wong, G., McCormick, F., and Williams, L. T. (1990). *Cell* **61,** 125–133.
Kaplan, K. B., Swedlow, J. R., Varmus, H. E., and Morgan, D. O. (1992). *J. Cell Biol.* **118,** 321–333.
Kaplan, K. B., Bibbins, K. B., Swedlow, J. R., Arnaud, M., Morgan, D. O., and Varmus, H. E. (1994). *EMBO J.* **13,** 4745–4756.
Kaplan, K. B., Swedlow, J. R., Morgan, D. O., and Varmus, H. E. (1995). *Genes Dev.* **9,** 1505–1517.
Kato, H., Faria, T. N., Stannard, B., Roberts, C. T., Jr., and LeRoith, D. (1994). *Mol. Endocrinol.* **8,** 40–50.
Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D., and Chambon, P. (1995). *Science* **270,** 1491–1494.
Kaufmann, A. M., Lichtner, R. B., Schirrmacher, V., and Khazaie, K. (1996). *Oncogene* **13,** 2349–2358.
Kauma, S. W., Aukerman, S. L., Eierman, D., and Turner, T. (1991). *J. Clin. Endocrinol. Metab.* **73,** 746–751.
Kazlauskas, A., and Cooper, J. A. (1989). *Cell* **58,** 1121–1133.
Kazlauskas, A., Ellis, C., Pawson, T., and Cooper, J. A. (1990). *Science* **247,** 1578–1581.
Khazaie, K., Schirrmacher, V., and Lichtner, R. B. (1993) *Cancer Metastasis Rev.* **12,** 255–274.
Kim, K., Akoto-Amanfu, E., Cherrick, H. M., and Park, N. H. (1991). *Oral Surg. Oral Med. Oral Pathol.* **71,** 303–311.
King, C. R., Swain, S. M., Porter, L., Steinberg, S. M., Lippmann, M. E., and Gelmann, E. P. (1989). *Cancer Res.* **49,** 4185–4191.
Klagsbrun, M., and D'Amore, P. A. (1996). *Cytokine Growth Factor Rev.* **7,** 259–270.
Klagsbrun, M., Knighton, D., and Folkman, J. (1976). *Cancer Res.* **36,** 110–114.
Klijn, J. G., Berns, E. M., Bontenbal, M., and Foekens, J. (1993). *Cancer Treat. Rev.* **19,** 45–63.
Klint, P., Kanda, S., and Claesson-Welsh, L. (1995). *J. Biol. Chem.* **270,** 23337–23344.
Kmiecik, T. E., and Shalloway, D. (1987). *Cell* **49,** 65–73.
Knighton, D. R., Zheng, J., Ten, E., Ashford, V. A., Xuong, N. H., Taylor, S. S., and Sowadski, J. M. (1991). *Science* **253,** 407–414.
Koenders, P. G., Beex, L. V., Geurts-Moespot, A., Heuvel, J. J., Kienhuis, C. B., and Benraad, T. J. (1991). *Cancer Res.* **51,** 4544–4548.
Koleske, A. J., Baltimore, D., and Lisanti, M. P. (1995). *Proc. Natl. Acad. Sci. U.S.A.* **92,** 1381–1385.
Komurasaki, T., Toyoda, H., Uchida, D., and Morimoto, S. (1997). *Oncogene* **15,** 2841–2848.
Koochekpour, S., Jeffers, M., Rulong, S., Taylor, G., Klineberg, E., Hudson, E. A., Resau, J. H., and Vande Woude, G. F. (1997). *Cancer Res.* **57,** 5391–5398.
Koster, A., Landgraf, S., Leipold, A., Sachse, R., Gebhart, E., Tulusan, A. H., Ronay, G., Schmidt, C., and Dingermann, T. (1991). *Anticancer Res.* **11,** 193–201.
Kraus, M. H., Popescu, N. C., Amsbaugh, S. C., and King, C. R. (1987). *EMBO J.* **6,** 605–610.
Kumble, S., Omary, M. B., Cartwright, C. A., and Triadafilopoulos, G. (1997). *Gastroenterology* **112,** 348–356.
Kumjian, D. A., Wahl, M. E., Rhee, S. G., and Daniel, T. O. (1989). *Proc. Natl. Acad. Sci. U.S.A.* **86,** 8232–8236.
Kundra, V., Escobedo, J. A., Kazlauskas, A., Kim, H. K., Rhee, S. G., Williams, L. T., and Zetter, B. R. (1994). *Nature (London)* **367,** 474–476.

Kypta, R. M., Goldberg, Y., Ulug, E. T., and Courtneidge, S. A. (1990). *Cell* **62,** 481–492.
Lamaze, C., Fujimoto, L. M., Yin, H. L., and Schmid, S. L. (1997). *J. Biol. Chem.* **272,** 20332–20335.
Langerak, A. W., De Laat, P. A., Van Der Linden, V., Delahaye, M., Van Der Kwast, T. H., Hoogsteden, H. C., Benner, R., and Versnel, M. A. (1996). *J. Pathol.* **178,** 151–160.
Lappi, DA. (1995). *Sem. Cancer Biol.* **6,** 279–288.
Lazennec, G., Ediger, T. R., Petz, L. N., Nardulli, A. M., and Katzenellenbogen B. S. (1997). *Mol. Endocrinol.* **11,** 1375–1386.
Lee, D., and Han, V. K. (1990). *In* "Handbook of Experimental Pharmacology," pp. 611–654. Springer-Verlag, Berlin and New York.
Lee, K. F., Simon, H., Chen, H., Bates, B., Hung, M. C., and Hauser, C. (1995). *Nature (London)* **378,** 394–398.
Lehrer, S., O'Shaughnessy, J., Song, H. K., Levine, E., Savoretti, P., Dalton, J., Lipsztein, R., Kalnicki, S., and Bloomer, W. D. (1989). *Mt. Sinai J. Med.* **56,** 83–85.
Lehtola, L., Partanen, J., Sistonen, L., Kohhonen, J., Warri, A., Harkonen, P., Clarke, R., and Alitalo, K. (1993). *Int. J. Cancer* **50,** 598–603.
Leiserowitz, G. S., Harris, S. A., Subramaniam, M., Keeney, G. I., Podratz, K. C., and Spelsberg, T. C. (1993). *Gynecol. Oncol.* **49,** 190–196.
Lemoine, N. R., Barnes, D. M., Hollywood, D. P., Hughes, C. M., Smith, P., Dublin, E., Prigent, S. A., Gullick, W. J., and Hurst, H. C. (1992). *Br. J. Cancer* **66,** 1116–1121.
Leon, S. P., Carroll, R. S., Dashner, K., Glowacka, D., and Black, P. M. (1994). *J. Clin. Endocrinol. Metab.* **79,** 51–55.
Levitzki, A., and Gazit, A. (1995). *Science* **267,** 1782–1788.
Levkowitz, G., Klapper, L. N., Tzahar, E., Freywald, A., Sela, M., and Yarden, Y. (1996). *Oncogene* **12,** 1117–1125.
Levy, P., Munier, A., Baron-Delage, S., Di Gioia, Y., Gespach, C., Capeau, J., and Cherqui, G. (1996). *Br. J. Cancer* **74,** 423–431.
Li, N., Batzer, A., Daly, R., Yajnik, V., Skolnik, E., Chardin, P., Bar-Sagi, D., Margolis, B., and Schlessinger, J. (1993). *Nature (London)* **363,** 85–88.
Li, S., Okamoto, T., Chun, M., Sargiacomo, M., Casanova, J. E., Hansen, S. H., Nishimoto, I., and Lisanti, M. P. (1995). *J. Biol. Chem.* **270,** 15693–15701.
Li, S., Couet, J., and Lisanti, M. P. (1996a). *J. Biol. Chem.* **271,** 29182–29190.
Li, S., Seitz, R., and Lisanti, M. P. (1996b). *J. Biol. Chem.* **271,** 3863–3868.
Libermann, T. A., Nusbaum, H. R., Rzaon, N., Kris, R., Lax, I., Soreq, H., Whittle, N., Waterfield, M. D., Ullrich, A., and Schlessinger, J. (1985). *Nature (London)* **313,** 144–147.
Libermann, T. A., Friesel, R., Jaye, M., Lyall, R. M., Westermark, B., Drohan, W., Schmidt, A., Maciag, T., and Schlessinger, J. (1987). *EMBO J.* **6,** 1627–1632.
Lichtner, R. B., Kaufmann, A. M., Kittmann, A., Rohde-Schulz, B., Walter, J., Williams, L., Ullrich, A., Schirrmacher, V., and Khazaie, K. (1995). *Oncogene* **10,** 1823–1832.
Lin, Y. J., Christianson, T. A., and Clinton, G. M. (1992). *J. Cell. Biochem.* **49,** 290–295.
Lisanti, M. P., Scherer, P. E., Vidugirene, J., Tang, Z., Hermanowski-Vosatka, A., Tu, Y. U., Cook, R. F., and Sargiacomo, M. (1994). *J. Cell Biol.* **126,** 111–126.
Liu, F., and Chernoff, J. (1997). *Biochem.* **327,** 139–145.
Longati, P., Bardelli, A., Ponzetto, C., Naldini, L., and Comoglio, P. M. (1994). *Oncogene* **9,** 49–57.
Longnecker, R. (1994). *Leukemia* **8,** S46–S50.
Lu, T. T., Yan, L. G., and Madri, J. A. (1996). *Proc. Natl. Acad. Sci. U.S.A.* **92,** 11808–11813.
Lu, T. T., Barreuther, M., Davis, S., and Madri, J. A. (1997). *J. Biol. Chem.* **272,** 14442–14446.
Lundy, J., Chen, J., Wang, P., Fromowitz, F., Schuss, A., Lynch, S., Brugge, J., and Viola, M. V. (1988). *Anticancer Res.* **8,** 1005–1013.
Luttrell, D. K., Luttrell, L. M., and Parsons, S. J. (1988). *Mol. Cell. Biol.* **8,** 497–501.
Luttrell, D. K., Lee, A., Lansing, T. J., Crosby, R. M., Jung, K. D., Willard, D., Luther, M.,

Rodriguez, J., Berman, J., and Gilmer, T. M. (1994). *Proc. Natl. Acad. Sci. U.S.A.* **91,** 83–87.
Maa, M. C., Wilson, L. K., Moyers, J. S., Vines, R. R., Parsons, J. T., and Parsons, S. J. (1992). *Proc. Natl. Acad. Sci. U.S.A.* **89,** 5720–5724.
Maa, M. C., Leu, T. H., McCarley, D. J., Schatzman, R. C., and Parsons, S. J. (1995). *Proc. Natl. Acad. Sci. U.S.A.* **92,** 6981–6985.
Malarkey, K., Belham, C. M., Graham, P. A., McLees, A., Scott, P. H., and Plevin, R. (1995). *Biochem. J.* **309,** 361–375.
Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995). *Cell* **83,** 835–839.
Mao, W., Irby, R., Coppola, D., Fu, L., Wloch, M., Turner, J., Yu, H., Garcia, R., Jove, R., and Yeatman, T. J. (1997). *Oncogene* **15,** 3083–3090.
Marchisio, P. C., Cirillo, D., Teti, A., Zambonin-Zallone, A., and Tarone, G. (1987). *Exp. Cell Res.* **169,** 202–214.
Matsuda, S., Kawamura-Tsuzuku, J., Ohsugi, M., Yoshida, M., Emi, M., Nakamura, Y., Onda, M., Yoshida, Y., Nishiyama, A., and Yamamoto, T. (1996). *Oncogene* **12** 705–713.
Matsui, T., Sano, K., Tsukamoto, T., Ito, M., Takaishi, T., Nakata, H., Nakamura, H., and Chihara, K. (1993). *J. Clin. Invest.* **92,** 1153–1160.
Matsumoto, K., Nakamura, T., and Kramer, R. H. (1994). *J. Biol. Chem.* **269,** 31807–31813.
Matten, W. T., Aubry, M., West, J., and Maness, P. F. (1990). *J. Cell Biol.* **111,** 1959–1970.
Mayer, B., and Hanafusa, H. (1990). *Proc. Natl. Acad. Sci. U.S.A.* **87,** 2638–2642.
Mazurenko, N. N., Kogen, E. A., Sukhova, N. M., and Zborovskaia, I. B. (1991a). *Vaprosy Med. Khimii* **37,** 53–59.
Mazurenko, N. N., Kogen, E. A., Zborovskaia, I. B., Sukhova, N. M., and Kiselev, F. L. (1991b). *Voprosy Onkol.* **37,** 683–690.
Meisenhelder, J., Suh, P. G., Rhee, S. G., and Hunter, T. (1989). *Cell* **57,** 1109–1122.
Meredith, S. D., Levine, P. A., Burns, J. A., Gaffey, M. J., Boyd, J. C., Weiss, L. M. Erickson, N. L., and Williams, M. E. (1995). *Arch. Otolaryngol.* **121,** 790–794.
Meyer, D., and Birchmeier, C. (1995). *Nature* **378,** 386–390.
Migliaccio, A., Di Domenico, M., Green, S., de Falco, A., Kajtaniak, E. L., Blasi, F., Chambon, P., and Auricchio, F. (1989). *Mol. Endocrinol.* **3,** 1061–1069.
Migliaccio, A., Pagano, M., and Auricchio, F. (1993). *Oncogene* **8,** 2183–2191.
Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E., and Auricchio, F. (1996). *EMBO J.* **15,** 1292–1300.
Mignatti, P., Tsuboi, R., Robbins, E., and Rifkin, D. B. (1989). *J. Cell Biol.* **108,** 671–682.
Min, H., Doyle, L., Vitt, C., Zandonella, C., Stratton-Thomas, J., Shuman, M., and Rosenberg, S. (1996). *Cancer Res.* **56,** 2428–2433.
Miyake, H., Yoshimura, K., Hara, I., Eto, H., Arakawa, S., and Kamidono, S. (1997). *J. Urol.* **157,** 2351–2355.
Miyoshi, N., Tateyama, S., Ogawa, K., Yamaguchi, R., Kuroda, H., Yasuda, N., and Shimizu, T. (1991). *Am. J. Vet. Res.* **52,** 2046–2049.
Moghul, A., Lin, L., Beedle, A., Kanbour-Shakir, A., DeFrances, M. C., Liu, Y., and Zarnegar, R. (1994). *Oncogene* **9,** 2045–2052.
Mohammadi, M., Dikic, I., Sorokin, A., Burgess, W. H., Jaye, M., and Schlessinger, J. (1996). *J. Virol.* **16,** 977–989.
Molloy, C. J., Bottaro, D. P., Fleming, T. P., Marshall, M. S., Gibbs, J. B., and Aaronson, S. A. (1989). *Nature (London)* **342,** 711–714.
Montesano, R., Vassalli, J. D., Baird, A., Guillemin, R., and Orci, L. (1986). *Proc. Natl. Acad. Sci. U.S.A.* **83,** 7297–7301.
Montgomery, R. B., Moscatello, D. K., Wong, A. J., Cooper, J. A., and Stahl, W. L. (1995). *J. Biol. Chem.* **270,** 30562–30566.

Mori, S., Ronnstrand, L., Yokote, K., Engstrom, A., Courtneidge, S. A., Claesson-Welsh, L., and Heldin, C. H. (1993). *EMBO J.* **12,** 2257–2264.
Morrison, D. K., Kaplan, D. R., Rhee, S. G., and Williams, L. T. (1990). *Mol. Cell. Biol.* **10,** 2359–2366.
Moscatello, D. K., Holgado-Madruga, M., Godwin, A. K., Ramirez, G., Gunn, G., Zoltick, P. W., Biegel, J. A., Hayes, R. L., and Wong, A. J. (1995). *Cancer Res.* **55,** 5536–5539.
Moscatello, D. K., Holgado-Madruga, M., Emlet, D. R., Montgomery, R. B., and Wong, A. J. (1998). *J. Biol. Chem.* **273,** 200–206.
Mukhopadhyay, D., Tsiokas, L., and Sukhatme, V. P. (1995a). *Cancer Res.* **55,** 6161–6165.
Mukhopadhyay, D., Tsiokas, L., Zhou, X. M., Foster, D., Brugge, J. S., and Sukhatme, V. P. (1995b). *Nature (London)* **375,** 577–581.
Muthuswamy, S. K., and Muller, W. J. (1995). *Oncogene* **11,** 271–279.
Muthuswamy, S. K., Siegel, P. M., Dankort, D. L., Webster, M. A., and Muller, W. J. (1994). *Mol. Cell. Biol.* **14,** 735–743.
Nada, S., Okada, M., Aizawa, S., and Nakagawa, H. (1994). *Oncogene* **9,** 3571–3578.
Nakamoto, T., Sakai, R., Ozawa, K., Yazaki, Y., and Hirai, H. (1996). *J. Biol. Chem.* **271,** 8959–8965.
Naldini, L., Weidner, K. M., Vigna, E., Gaudino, G., Bardelli, A., Ponzetto, C., Narsimhan, R. P., Hartmann, G., Zarnegar, R., Michalopoulos, G. K., and Birchmeier, W., and Comoglio, P. M. (1991). *EMBO J.* **10,** 2867–2878.
Nelson, K. G., Takahashi, T., Bossert, N. L., Walmer, D. K., and McLachlan, J. A. (1991). *Proc. Natl. Acad. Sci. U.S.A.* **88,** 21–25.
Newman, P. J., Berndt, M. C., Gorski, J., White, G. C., II., Lyman, S., Paddock, C., and Muller, W. A. (1990). *Science* **247,** 1219–1222.
Nexo, E., and Kryger-Baggesen, N. (1989). *Regul. Pept.* **26,** 1–8.
Nguyen, M., Watanabe, H., Budson, A. E., Richie, J. P., Hayes, D. F., and Folkman, J. (1994). *J. Natl. Cancer Inst.* **86,** 356–361.
Nicholson, S., Halcrow, P., Sainsbury, J. R., Angus, B., Chambers, P., Farndon, J. R., and Harris, A. L. (1988). *Br. J. Cancer* **58,** 810–814.
Nicholson, S., Wright, C., Sainsbury, J. R., Halcrow, P., Kelly, P., Angus, B., Farndon, J. R., and Harris, A. L. (1990). *J. Steroid Biochem. Mol. Biol.* **37,** 811–814.
Nojima, Y., Morino, N., Mimura, T., Hamasaki, K., Furuya, H., Sakai, R., Sato, T., Tachibana, K., Morimoto, C., and Yazaki, Y. (1995). *J. Biol. Chem.* **270,** 15398–15402.
Novak, U., Nice, E., Hamilton, J. A., and Paradiso, L. (1996). *Oncogene* **13,** 2607–2613.
Novotny-Smith, C. L., and Gallick, G. E. (1992). *J. Immunother.* **11,** 159–168.
Okada, M., Nada, S., Yamanashi, Y., Yamamoto, T., and Nakagawa, H. (1991). *J. Biol. Chem.* **266,** 24249–24252.
Okamura, H., and Resh, M. D. (1995). *J. Biol. Chem.* **270,** 26613–26618.
Onoda, T., Iinuma, H., Sasaki, Y., Hamada, M., Isshiki, K., Naganawa, H., Takeuchi, T., Tatsuta, K., and Umezawa, K. (1989). *J. Nat. Prod.* **52,** 1252–1257.
Osherov, N., and Levitzki, A. (1994). *Eur. J. Biochem.* **225,** 1047–1053.
Osornio-Vargas, A. R., Lindroos, P. M., Coin, P. G., Badgett, A., Hernandez-Rodriguez, N. A., and Bonner, J. C. (1996). *Am. J. Physiol.* **271,** L93–L99.
Ottenhoff-Kalff, A. E., Rijksen, G., van Beurden, E. A., Hennipman, A., Michels, A. A., and Staal, G. E. (1992). *Cancer Res.* **52,** 4773–4778.
Oude Weernink, P. A., Ottenhoff-Kalff, A. E., Vandrig, M. P., van Beurden, E. A., Staal, G. E., and Rijksen, G. (1994). *FEBS Lett.* **352,** 296–300.
Owens, L. V., Xu, L., Craven, R. J., Dent, G. A., Weiner, T. M., Kornberg, L., Liu, E. T., and Cance, W. G. (1995). *Cancer Res.* **55,** 2752–2755.
Paech, K., Webb, P., Kuiper, G. G., Nilsson, S., Gustafsson, J. A., Kushner, P. J., and Scanlan, T. S. (1997). *Science* **277,** 1508–1510.

Paietta, E., Racevsbis, J., Stanley, E. R., Andreef, M., Papenhausen, P., and Wiernik, P. H. (1990). *Cancer Res.* **50,** 2049–2055.
Paik, S., Hazan, R., Fisher, E. R., Sass, R. E., Fisher, B., Redmond, C., Schlessinger, J., Lippman, M. E., and King, C. R. (1990). *J. Clin. Oncol.* **8,** 103–112.
Park, M., Dean, M., Kaul, K., Braun, M. J., Gonda, M. A., and Vande Woude, G. F. (1987). *Proc. Natl. Acad. Sci. U.S.A.* **84,** 6379–6383.
Parsons, S. J., and Creutz, C. E. (1986). *Biochem. Biophys. Res. Commun.* **134,** 736–742.
Parsons, J. T., and Parsons, S. J. (1997). *Curr. Opin. Cell Biol.* **9,** 187–192.
Pastone, G., Pasquale, E. B., and Maher, P. A. (1993). *Dev. Biol.* **155,** 107–123.
Pawson, T., and Schlessinger, J. (1993). *Curr. Biol.* **3,** 434–442.
Pelicci, G., Lanfrancona, L., and Grignani, F. (1992). *Cell* **70,** 93–104.
Pena, S. V., Melhem, M. F., Meisler, A. I., and Cartwright, C. A. (1995). *Gastroenterology* **108,** 117–124.
Penault-Llorca, F., Bertucci, F., Adelaide, J., Parc, P., Coulier, F., Lacquemier, J., Birnbaum, D., and DeLapeyriere, O. (1995). *Int. J. Cancer* **61,** 170–176.
Petch, L. A., Bockholt, S. M., Bouton, A., Parsons, J. T., and Burridge, K. (1995). *J. Cell Sci.* **108,** 1371–1379.
Peters, K., Werner, S., Chen, G., and Williams, L. (1992). *Development* **114,** 233–243.
Peters, K., Ornitz, D., Werner, S., and Williams, L. (1993). *Dev. Biol.* **155,** 423–430.
Pietras, R. J., Arboleda, J., Reese, D. M., Wongvipat, N., Pegram, M. D., Ramos, L., Gorman, C. M., Parker, M. G. Sliwkowski, M. X., and Slamon, D. J. (1995). *Oncogene* **10,** 2435–2446.
Pinkas-Kramarski, R., Soussan, L., Waterman, H., Levkowitz, G., Alroy, I., Klapper, L., Lavi, S., Seger, R., Ratzkin, B. J., Sela, M., and Yarden, Y. (1996). *EMBO J.* **15,** 2542–2467.
Pinter, E., Barreuther, M., Lu, T., Imhof, B. A., and Madri, J. A. (1997). *Am. J. Pathol.* **150,** 1523–1530.
Piwnica-Worms, H., Saunders, K. B., Roberts, T. M., Smith, A. E., and Cheng, S. H. (1987). *Cell* **49,** 75–82.
Plummer, M. S., Lunney, E. A., Para, K. S., Prasad, J. V., Shahripour, A., Singh, J., Stankovic, C. J., Humblet, C., Fergus, J. H., Marks, J. S., and Sawyer, T. K. (1996). *Drug Des. Discov.* **13,** 75–81.
Plummer, M. S., Lunney, E. A., Para, K. S., Shahripour, A., Stankovic, C. J., Humblet, C., Fergus, J. H., Marks, J. S., Herrera, R., Hubbell, S., Saltiel, A., and Sawyer, T. K. (1997) . *Bioorg. Med. Chem.* **5,** 41–47.
Pollard, J. W., Bartocci, A., Arceci, R., Orlofsky, A., and Ladner, M. B. (1987). *Nature* **330,** 484–486.
Polte, T. R., and Hanks, S. K. (1995). *Proc. Natl. Acad. Sci. U.S.A.* **92,** 10678–10682.
Ponten, F., Ren, Z., Nister, M., Westermark, B., and Ponten, J. (1994). *J. Invest. Dermatol.* **102,** 304–309.
Ponzetto, C., Bardelli, A., Zhen, Z., Maina, F., dalla Zonca, P., Giordano, S., Graziani, A., Panayotou, G., and Comoglio, P. M. (1994). *Cell* **77,** 261–271.
Potapova, O., Fakhrai, H., Baird, S., and Mercola, D. (1996). *Cancer Res.* **56,** 280–286.
Press, M. F., Cordon-Cardo, C., and Slamon, D. J. (1990). *Oncogene* **5,** 953–962.
Rak, J., Filmus, J., Finkenzeller, G., Grugel, S., Marme, D., and Kerbel, R. S. (1995). *Cancer Metastasis Rev.* **14,** 263–277.
Ralston, R., and Bishop, J. M. (1985). *Proc. Natl. Acad. Sci. U.S.A.* **82,** 7845–7849.
Rambaldi, A., Wakamiya, N., Vellenga, E., Horiguchi, J., Warren, M. K., Kufe, D., and Griffin, J. D. (1988). *J. Clin. Invest.* **81,** 1030–1035.
Rappoll, D., Brenner, C. A., Schultz, R., Mark, D., and Werb, Z. (1988). *Science* **241,** 1823–1825.
Reedijk, M., Liu, X. Q., and Pawson, T. (1990). *Mol. Cell. Biol.* **10,** 5601–5608.

Regensstreif, L., and Rossant, J. (1989). *Dev. Biol.* **133**, 284–294.
Resh, M. D. (1994). *Cell* **76**, 411–413.
Rettenmeier, C. W., Roussel, M. F., Ashmun, R. A., Ralph, P., Price, K., and Sherr, C. J. (1987). *J. Virol.* **7**, 2378–2387.
Reynolds, A. B., Vila, J., Lansing, T. J., Potts, W. M., Weber, M. J., and Parsons, J. T. (1987). *EMBO J.* **6**, 2359–2364.
Rhee, S. G. (1991). *Trends Biochem. Sci.* **16**, 297–301.
Ridley, A. J., and Hall, A. (1992). *Cell* **70**, 401–410.
Riese, I. I., Bermingham, Y., van Raaij, T. M., Buckley, S., Plowman, G. D., and Stern, D. F. (1996). *Oncogene* **12**, 345–353.
Roche, S., Koegl, M., Barone, M. V., Roussel, M. F., and Courtneidge, S. A. (1995). *Mol. Cell. Biol.* **15**, 1102–1109.
Romano, A., Wong, W. T., Santoro, M., Wirth, P. J., Thorgeirsson, S. S., and Di Fiore, P. P. (1994). *Oncogene* **9**, 2923–2933.
Ron, D., Reich, R., Chedid, M., Lengel, C., Cohen, O., Chen, A., Neufeld, G., Miki, T., and Tronick, S. (1993). *J. Biol. Chem.* **268**, 5388–5394.
Rong, S., Bodescot, M., Blair, D., Dunn, J., Nakamura, T., Mizuno, K., Park, M., Chan, A., Aaronson, S., and Vande Woude, G. F. (1992). *Mol. Cell. Biol.* **12**, 5152–5158.
Ronsin, C., Muscatelli, F., Mattei, M. G., and Breathnach, R. (1993). *Oncogene* **8**, 1195–1202.
Roof, R. W., Haskell, M. D., Sherman, N., Kinter, M., and Parsons, S. J. (1998). Submitted.
Rosen, E. M., and Goldberg, I. D. (1995). *Adv. Cancer Res.* **67**, 257–279.
Rosen, N., Bolen, J. B., Schwartz, A. M., Cohen, P., DeSeau, V., and Israel, M. A. (1986). *J. Biol. Chem.* **261**, 13754–13759.
Rosen, E. M., Knesel, J., Goldberg, I. D., Bhargava, M., Joseph, A., Zitnik, R., Wines, J., Kelley, M., and Rockwell, S. (1994). *Int. J. Cancer* **57**, 706–714.
Rosnet, O., and Birnbaum, D. (1993). *Crit. Rev. Oncogen.* **4**, 595–613.
Ross, R., Bowen-Pope, D. F., and Raines, E. W. (1990). *Philos. Trans. R. Soc. London [Biol.]* **327**, 155–169.
Roth, P., and Stanley, E. R. (1992). *Curr. Top. Microbiol. Immunol.* **181**, 141–167.
Roulston, D., Espinosa, I. I., Stoffel, M., Bell, G. I., and Le Beau, M. M. (1993). *Blood* **82**, 3424–3429.
Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T., and Bowtell, D. (1993). *Nature (London)* **363**, 83–85.
Rubin, J. S., Chan, A. M., Bottaro, D. P., Burgess, W. H., Taylor, W. G., Cech, A. C., Hirschfield, D. W., Wong, J., Miki, T., Finch, P. W., and Aaronson, S. A. (1991). *Proc. Natl. Acad. Sci. U.S.A.* **88**, 415–419.
Ruff-Jamison, S., McGlade, J., Pawson, T., Chen, K., and Cohen, S. (1993). *J. Biol. Chem.* **268**, 7610–7612.
Russo, A. A., Jeffrey, P. D., and Pavletich, N. P. (1996). *Nature Struct. Biol.* **3**, 696–700.
Sager, R., Sheng, S., Anisowicz, A., Sotiropoulou, G., Zou, Z., Stenman, G., Swisshelm, K., Chen, Z., Hendrix, M. J., Pemberton, P., Rafidi, K., and Ryan, K. (1994). *Cold Spring Harb. Symp. Quant. Biol.* **59**, 537–546.
Sainsbury, J. R., Farndon, J. R., Sherbet, G. V., and Harris, A. L. (1985). *Lancet* **1**, 364–366.
Sainsbury, J. R., Farndon, J. R., Needham, G. K., Malcolm, A. J., and Harris, A. L. (1987). *Lancet* **1**, 1398–1402.
Salgia, R., Avraham, S., Pisick, E., Li, J. L., Raja, S., Greenfield, E. A., Sattler, M., Avraham, H., and Griffin, J. D. (1996). *J. Biol. Chem.* **271**, 31222–31226.
Sargiacomo, M., Sudol, M., Tang, Z., and Lisanti, M. P. (1993). *J. Cell Biol.* **122**, 789–807.
Sato, Y., and Rifkin, D. B. (1988). *J. Cell Biol.* **107**, 1199–1205.
Sato, K. I., Sato, A., Aoto, M., and Fukami, Y. (1995). *Biochem. Biophys. Res. Commun.* **215**, 1078–1087.

Scambia, G., Benedetti-Panici, P., Ferrandina, G., Distefano, M., Salerno, G., Romanini, M. E., Fagotti, A., and Mancuso, S. (1995). *Br. J. Cancer* **72,** 361–366.
Schaller, M., and Parsons, J. T. (1994). *Curr. Opin. Cell Biol.* **6,** 705–710.
Schaller, M. D., Hildebrand, J. D., Shannon, J. D,., Fox, J. W., Vines, R. R., and Parsons, J. T. (1994). *J. Virol.* **14,** 1680–1688.
Schimmenti, L. A., Yan, H. C., Madri, J. A., and Albelda, S. M. (1992). *J. Cell. Physiol.* **153,** 417–428.
Schlaepfer, D. D., and Hunter, T. (1997). *J. Biol. Chem.* **272,** 13189–13195.
Schlaepfer, D. D., Broome, M. A., and Hunter, T. (1997). *J. Virol.* **17,** 1702–1713.
Schmidt, L., Duh, F. M., Chen, F., Kishida, T., Glenn, G., Choyke, P., Schere, S. W., Zhuang, Z., Lubensky, I., Dean, M., Allikmets, R., Chidambaram, A., Bergerheim, U. R., Feltis, J. T., Casadevall, C., Zamarron, A., Bernues, M., Richard, S., Lips, C. J., Walther, M. M., Tsui, L. C., Geil, L., Orcutt, M. L., Stackhouse, T., and Zbar, B. (1997). *Nature Genet.* **16,** 68–73.
Schubert, D., Ling, N., and Baird, A. (1987) *J. Cell Biol.* **104,** 635–643.
Schuuring, E., Verhoeven, E., Mooi, W. J., and Michalides, R. J. (1992). *Oncogene* **7,** 355–361.
Schuuring, E., Verhoeven, E., Litvinov, S., and Michalides, R. J. (1993). *Mol. Cell. Biol.* **13,** 2891–2898.
Schweigerer, L., Neufeld, G., and Gospodarowicz, D. (1987). *J. Clin. Invest.* **80,** 1516–1520.
Seki, T., Fujii, G., Mori, S., Tamaoki, N., and Shibuya, M. (1985). *Jpn. J. Cancer Res.* **76,** 907–910.
Senior, R. M., Huang, S. S., Griffin, G. L., and Huang, J. S. (1986). *Biochem. Biophys. Res. Commun.* **141,** 67–72.
Sepp-Lorenzino, L., Ma, Z., Lebwohl, D. E., Vinitsky, A., and Rosen, N. (1995). *J. Biol. Chem.* **270,** 16580–16587.
Settleman, J., Albright, C. F., Foster, L. C., and Weinberg, R. A. (1992a). *Nature (London)* **359,** 153–154.
Settleman, J., Narasimhan, V., Foster, L. C., and Weinberg, R. A. (1992b). *Cell* **69,** 539–549.
Seufferlein, T., and Rozengurt, E. (1994). *J. Biol. Chem.* **269,** 27610–27617.
Shalloway, D., Coussens, P. M., and Yaciuk, P. (1984). *Proc. Natl. Acad. Sci. U.S.A.* **81,** 7071–7075.
Shapiro, R., Duquette, J., Roses, D., Nunes, I., Harris, M., Kamino, H., Wilson, E., and Rifkin, D. (1996). *Cancer Res.* **56,** 3597–3604.
Sherr, C. J. (1990). *Blood* **75,** 1–12.
Sherr, C. J., Rettenmier, C. W., Sacca, R., Roussel, M. F., Look, A. T., and Stanley, E. R. (1985). *Cell* **41,** 665–676.
Shinbrot, E., Peters, K. G., and Williams, L. T. (1994). *Dev. Dynam.* **199,** 169–175.
Shing, Y., Christofori, G., Hanahan, D., Ono, Y., Sasada, R., Igarashi, K., and Folkman, J. (1993). *Science* **259,** 1604–1607.
Shoji, S., Kurosawa, T., Inoue, H., Funakoshi, T., and Kubota, Y. (1990). *Biochem. Biophys. Res. Commun.* **173** 894–901.
Shurtleff, S. A., Downing, J. R., Rock, C. O., Hawkins, S. A., Roussel, M. F., and Sherr, C. J. (1990). *EMBO J.* **9,** 2415–2421.
Sicheri, F., Moarefi, I., and Kuriyan, J. (1997). *Nature (London)* **385,** 602–609.
Simpson, N. E. (1988). *J. Med. Genet.* **25,** 794–804.
Slamon, D. J., deKernion, J. B., Verma, I. M., and Cline, M. J. (1984). *Science* **224,** 256–262.
Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., and Press, M. F. (1989). *Science* **244,** 707–712.
Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. (1987). *Science* **235,** 177–182.
Slavin, J. (1995). *Cell Biol. Int.* **19,** 431–444.
Smithgall, T. E. (1995). *J. Pharmacol. Toxicol. Methods* **34,** 125–132.

Soltesz, S. A., Powers, E. A., Geng, J. G., and Fisher, C. (1997). *Int. J. Cancer* **71**, 645–653.
Soriano, P. (1997). *Development* **124**, 2691–2700.
Sorkin, A., and Carpenter, G. (1993). *Science* **261**, 612–615.
Souza, P., Kuliszewski, M., Wang, J., Tseu, I., Tanswell, A. K., and Post, M. (1995). *Development* **121**, 2559–2567.
Sporn, M., and Roberts, A. (1988). *Nature (London)* **332**, 217–219.
Stacey, K. J., Fowles, L. F., Colman, M. S., Ostrowski, M. J., and Hume, D. A. (1995). *J. Virol.* **15**, 3430–3441.
Staley, C. A., Parikh, N. J., and Gallick, G. E. (1997). *Cell Growth Differ.* **8**, 269–274.
Storga, D., Pecina-Slaus, N., Pavelic, J., Pavelic, Z. P., and Pavelic, K. (1992). *Int. J. Exp. Pathol.* **73**, 527–533.
Stover, D. R., Becker, M., Liebetanz, J., and Lydon, N. B. (1995). *J. Biol. Chem.* **270**, 15591–15597.
Sudol, M., Grant, S. G., and Maisonpierre, P. C. (1993). *Neurochem. Int.* **22**, 369–384.
Takekura, N., Yasui, W., Yoshida, K., Tsujino, T., Nakayama, H., Kameda, T., Yokozaki, H., Nishimura, Y., Ito, H., and Tahara, E. (1990). *Int. J. Cancer* **45**, 847–851.
Takes, R. P., Baatenburg, d. J., Schuuring, E., Hermans, J., Vis, A. A., Litvinov, S. V., and van Krieken, J. H. (1997). *Arch. Otolaryngol.* **123**, 412–419.
Talamonti, M. S., Roh, M. S., Curley, S. A., and Gallick, G. E. (1993). *J. Clin. Invest.* **91**, 53–60.
Tanaka, Y., Albelda, S. M., Horgan, K. J., van Seventer, G. A. Shimizu, Y., Newman, W., Hallam, J., Newman, P. J., Buck, C. A., and Shaw, S. (1992). *J. Exp. Med.* **176**, 245–253.
Tang, R., Beuvon, F., Ojeda, M., Mosseri, V., Pouillart, P., and Scholl, S. (1992). *J. Cell Biol.* **50**, 350–356.
Taylor, G. R., Reedijk, M., Rothwell, V., Rohrschneider, L., and Pawson, T. (1989). *EMBO J.* **8**, 2029–2037.
Thomas, S. M., Soriano, P., and Imamoto, A. (1995). *Nature (London)* **376**, 267–271.
Tice, D. A., Biscardi, J. S., Nickles, A. L., and Parsons, S. J. (1998). Submitted.
Till, K. J., Lopez, A., Slupsky, J., and Cawley, J. C. (1993). *Br. J. Haematol.* **83**, 223–231.
Tkachuk, V., Stepanova, V., Little, P. J., and Bobik, A. (1996). *Clin. Exp. Pharmacol. Physiol.* **23**, 759–765.
Toi, M., Osaki, A., Yamada, H., and Toge, T. (1991). *Eur. J. Cancer* **27**, 977–980.
Toshima, J., Ohashi, K., Iwashita, S., and Mizuno, K. (1995). *Biochem. Biophys. Res. Commun.* **209**, 656–663.
Traxler, P., Furet, P., Mett, H., Buchdunger, E., Meyer, T., and Lydon, N. (1997). *J. Pharm. Belg.* **52**, 88–96.
Tremblay, L., Hauck, W., Aprikian, A. G., Begin, L. R., Chapdelaine, A., and Chevalier, S. (1996). *Int. J. Cancer* **68**, 164–171.
Tsai, M. J., and O'Malley, B. W. (1994). *Annu. Rev. Biochem.* **63**, 451–486.
Tuzi, N. L., Venter, D. J., Kumar, S., Staddon, S. L., Lemoine, N. R., and Gullick, W. J. (1991). *Br. J. Cancer* **63**, 227–233.
Twamley, G. M., Kypta, R. M., Hall, B., and Courtneidge, S. A. (1992). *Oncogene* **7**, 1893–1901.
Twamley-Stein, G. M., Pepperkok, R., Ansorge, W., and Courtneidge, S. A. (1993). *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7696–7700.
Uehara, Y., Hori, M., Takeuchi, T., and Umezawa, H. (1986). *Mol. Cell. Biol.* **6**, 2198–2206.
Uehara, Y., Fukazawa, H., Murakami, Y., and Mizuno, S. (1989a). *Biochem. Biophys. Res. Commun.* **163** 803–809.
Uehara, Y., Murakami, Y., Sugimoto, Y., and Mizuno, S. (1989b). *Cancer Res.* **49**, 780–785.
Ullrich, A., and Schlessinger, J. (1990). *Cell* **61**, 203–212.
Valius, M., and Kazlauskas, A. (1993). *Cell* **73**, 321–334.

van Damme, H., Brok, H., Schuuring-Scholtes, E., and Schurring, E. (1997). *J. Biol. Chem.* **272,** 7374–7380.
van der Geer, P., and Hunter, T. (1989). *J. Virol.* **9,** 1336–1341.
van der Geer, P., and Hunter, T. (1991). *Mol. Cell. Biol.* **11,** 4698–4709.
van de Vijver, M. J., Peterse, J. L., Mooi, W. J., Wisman, P., Lomans, J., Dalesio, O., and Nusse, R. (1988). *N. Engl. J. Med.* **319** 1239–1245.
van Hoek, M. L., Allen, C. S., and Parsons, S. J. (1997). *Biochem. J.* **326,** 271–277.
van Puijenbroek, A. A., van Weering, D. H., van den Brink, C. E., Bos, J. L., van der Saag, P. T., de Laat, S. W., and den Hertog, J. (1997). *Oncogene* **14,** 1147–1157.
Varticovski, L., Druker, B., Morrison, D., Cantley, L., and Roberts, T. (1989). *Nature (London)* **342,** 699–702.
Velu, T. J., Beguinot, L., Vass, W. C., Willingham, M. C., Merlino, G. T., Pastan, I., and Lowy, D. R. (1987). *Science* **238,** 1408–1410.
Verbeek, B. S., Vroom, T. M., Adriaansen-Slot, S. S., Ottenhoff-Kalff, A. E., Geertzema, J. G., Hennipman, A., and Rijksen, G. (1996). *J. Pathol.* **180,** 383–388.
Versnel, M. A., Haarbrink, M., Langerak, A. W., De Laat, P. A., Hagemeijer, A., Van Der Kwast, T. H., van den Berg, B., and Schrier, P. I. (1994). *Cancer Genet. Cytogenet.* **73,** 60–64.
Vignaud, J. M., Marie, B., Klein, N., Plenat, F., Pech, M., Borrelly, J., Martinet, N., Duprez, A., and Martinet, Y. (1994). *Cancer Res.* **54,** 5455–5463.
Vuori, K., Hirai, H., Aizawa, S., and Ruoslahti, E. (1996). *J. Virol.* **16,** 2606–2613.
Wadzinski, M., Folkman, J., Sasse, J., Devey, K., Ingber, D., and Klagsbrun, M. (1987). *Clin. Physiol. Biochem.* **5,** 200–209.
Wahl, M. I., Olashaw, N. E., Nishibe, S., Rhee, S. G., Pledger, W. J., and Carpenter, G. (1989). *Mol. Cell. Biol.* **9,** 2934–2943.
Walicke, P. A. (1988). *J. Neurosci* **8,** 2618–2627.
Walker, F., deBlaquiere, J., and Burgess, A. W. (1993). *J. Biol. Chem.* **268,** 19552–19558.
Wanaka, A., Milbrandt, J., and Johnson, E. (1991). *Development* **111,** 455–468.
Wang, Z., and Moran, M. F. (1996). *Science* **272,** 1935–1939.
Wang, P., Fromowitz, F., Koslow, M., Hagag, N., Johnson, B., and Viola, M. (1991). *Br. J. Cancer* **64,** 531–533.
Wang, F., Kan, M., Xu, J., Yan, G., and McKeehan, W. L. (1995). *J. Biol. Chem.* **270,** 10222–10230.
Wantanabe, N., Matsuda, S., Kuramochi, S., Tsuzuku, J., Yamamoto, T., and Endo, K. (1995). *Jpn. J. Clin. Oncol.* **25,** 5–9.
Ware, M. F., Tice, D. A., Parsons, S. J., and Lauffenburger, D. A. (1997). *J. Biol. Chem.* **272,** 30185–30190.
Webster, M. A., Cardiff, R. D., and Muller, W. J. (1995). *Proc. Natl. Acad. Sci. U.S.A.* **92,** 7849–7853.
Weidner, K. M., Sachs, M., and Birchmeier, W. (1993). *J. Cell Biol.* **121,** 145–154.
Weiner, T. M., Liu, E. T., Craven, R. J., and Cance, W. G. (1994). *Ann. Surg. Oncol.* **1,** 18–27.
Weis, K. E., Ekena, K., Thomas, J. A., Lazennec, G., and Katzenellenbogen, B. S. (1996). *Mol. Endocrinol.* **10,** 1389–1398.
Weiss, F. U., Daub, H., and Ullrich, A. (1997). *Curr. Opin. Genet. Dev.* **7,** 80–86.
Weissenberger, J., Steinbach, J. P., Malin, G., Spada, S., Rulicke, T., and Aguzzi, A. (1997). *Oncogene* **14,** 2005–2013.
Wellbrook, C., Lammers, R., Ullrich, A., and Schartl, M. (1995). *Oncogene* **10,** 2135–2143.
Wiktor-Jedrzejczak, W., Bartocci, A., Ferrante, J., Ahmed-Ansari, A., Sell, K. W., Pollard, J. W., and Stanley, E. R. (1990). *Proc. Natl. Acad. Sci. U.S.A.* **87,** 4828–4832.
Wiktor-Jedrzejczak, W., Ubranowska, E., Aukerman, S. L., Pollard, J. W., Stanley, E. R., Ralph, P., Ansari, A. A., Sell, K. W., and Szperl, M. (1991). *Proc. Natl. Acad. Sci. U.S.A.* **19,** 1049–1054.

Williams, M. E., Gaffey, M. J., Weiss, L. M., Wilczynski, S. P., Schuuring, E., and Levine, P. A. (1993). *Arch. Otolaryngol.* **119**, 1238–1243.
Wilson, L. K., and Parsons, S. J. (1999). Oncogene **5**, 1471–1480.
Wilson, L. K., Luttrell, D. K., Parsons, J. T., and Parsons, S. J. (1989). *Mol. Cell. Biol.* **9**, 1536–1544.
Withers, B. E., Hanks, S. K., and Fry, D. W. (1996). *Cancer Biochem. Biophys.* **15**,127–139.
Wong, A. J., Ruppert, J. M. Bigner, S. H., Grzeschik, C. H., Humphrey, P. A. Bigner, D. S., and Vogelstein, B. (1992). *Proc. Natl. Acad. Sci. U.S.A.* **89**, 2965–2969.
Woolford, J., Rothwell, V., and Rohrschneider, L. (1985). *J. Virol.* **5**, 3458–3466.
Wright, C., Nicholson, S., Angus, B., Sainsbury, J. R., Farndon, J., Cairns, J. Harris, A. L., and Horne, C. H. (1992). *Br. J. Cancer* **65**, 118–121.
Wu, H., and Parsons, J. T. (1993). *J. Cell Biol.* **120**, 1417–1426.
Wu, H., Reynolds, A. B., Kanner, S. B., Vines, R. R., and Parsons, J. T. (1991). *J. Virol.* **11**, 5113–5124.
Xian, W., Rosenberg, M. P., and DiGiovanni, J. (1997). *Oncogene* **14**, 1435–1444.
Xu, W., Harrison, S. C., and Eck, M. J. (1997). *Nature (London)* **385**, 595–602.
Yamaguchi, H., and Hendrickson, W. A. (1996). *Nature (London)* **384**, 484–489.
Yamashita, J., Ogawa, M., Yamashita, S., Nomura, K., Kuramoto, M., Saishoji, T., and Sadahito, S. (1994). *Cancer Res.* **54**, 1630–1633.
Yamazaki, H., Yasuhisa, F., Ueyama, Y., Tamaoli, N., Kawamoto, T., Taniguchi, S., and Shibuya, M. (1988). *J. Virol.* **8**, 1816–1820.
Yan, H. C., Baldwin, H. S., Sun, J., Buck, C. A., Albelda, S. M., and DeLisser, H. M. (1995). *J. Biol. Chem.* **270**, 23672–23680.
Yarden, Y., Escobedo, J. A., Kuang, W. J., Yang-Feng, T. L., Daniel, T. O., Tremble, P. M., Chen, E. Y., Ando, M. E., Harkins, R. N., Francke, U., and Fried, V. A., Ullrich A., and Williams, L. T. (1986). *Nature (London)* **323**, 226–232.
Yarden, R. I., Lauber, A. H., El-Ashry, D., and Chrysogelos, S. A. (1996). *Endocrinology* **137**, 2739–2747.
Yayon, A., Ma, Y. S., Safran, M., Klagsbrun, M., and Halaban, R. (1997). *Oncogene* **14**, 2999–3009.
Yokote, K., Mori, S., Siegbahn, A., Ronnstrand, L., Wernstedt, C., Heldin, C. H., and Claesson-Welsh, L. (1996). *J. Biol. Chem.* **271**, 5101–5111.
Yu, X. M., Askalan, R., Keil, G. J., and Salter, M. W. (1997). *Science* **275**, 674–678.
Zachary, I., Sinnett-Smith, J., and Rozengurt, E. (1992). *J. Biol. Chem.* **267**, 19031–19034.
Zhan, X., Hu, X., Friesel, R., and Maciag, T. (1993). *J. Biol. Chem.* **268**, 9611–9620.
Zhan, X., Plourde, Hu, X., Friesel, R., and Maciag, T. (1994). *J. Biol. Chem.* **269**, 20221–20224.
Zhang, Y., Paria, B. C., Dey, S. K., and Davis, D. L. (1992). *Dev. Biol.* **151**, 617–621.
Zhau, H. Y., Zhou, J., Symmans, W. F., Chen, B. Q., Chang, S. M., Sikes, R. A., and Chung, L. W. (1996). *Prostate* **28**, 73–83.
Zhu, H., Naujokas, M. A., and Park, M. (1994). *Cell Growth Differ.* **5**, 359–366.

THE CARBOXY-TERMINUS OF C-SRC INHIBITS BREAST TUMOR CELL GROWTH BY A KINASE-INDEPENDENT MECHANISM

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Running Title: c-Src is required for breast tumor growth.

ABSTRACT

Cellular Src (c-Src) is frequently overexpressed or activated in human breast cancer. However, the functional significance of this overexpression for the human disease has not been determined. Here, we show that growth in soft agar or tumorigenicity in nude mice of several human breast tumor cell lines is inhibited by either a Src family inhibitor, PP1, or stable overexpression of a kinase-inactive c-Src mutant, suggesting that c-Src is required for breast tumor cells to maintain a tumorigenic phenotype. To determine which domain(s) of c-Src is responsible for inhibiting tumorigenicity, we generated MDA-MB-468 breast tumor cells which stably or transiently overexpress each of the c-Src domains. While the unique, Src-homology domain 3, and amino-terminal half of c-Src had little to no effect, the Src-homology domain 2 and surprisingly a kinase inactive form of the carboxy-terminal half inhibited DNA synthesis and soft agar growth of MDA-MB-468 breast tumor cells. Furthermore, the carboxy-terminal half of c-Src was found to co-precipitate with an unidentified protein of approximately 95 kDa, suggesting a protein interaction function of the carboxy-terminus in addition to its kinase activity. These results implicate the carboxy-terminal half of c-Src as containing two potential therapeutic targets for breast cancer.

Introduction

The ubiquitously expressed tyrosine kinase c-Src is overexpressed or activated in a number of human tumors including carcinomas of the breast, colon, lung, skin, esophagus, cervix, and gastric tissues as well as in the development of neuroblastomas and myeloproliferative disorders (3). These findings raise the question as to whether c-Src contributes to the tumorigenic phenotype. Attempts to address this issue in tissue culture model systems have revealed that overexpression of c-Src alone is non- or weakly oncogenic (21, 42), suggesting that aberrant overexpression of the enzyme by itself is not a causative tumorigenic agent.

However, it has been shown that co-overexpression of c-Src with the epidermal growth factor receptor (EGFR) can potentiate EGF-induced mitogenesis and tumorigenesis in murine fibroblasts (3, 24, 44). An analysis of a panel of human breast tumor cell lines and tumor tissues supported the hypothesis that c-Src/EGFR interactions contribute to tumor progression and demonstrated the relevance of this interaction for human neoplasias (2). This potentiation is dependent upon a c-Src-mediated phosphorylation of Tyr 845 on the EGFR. The phosphorylation on Tyr 845 is required not only for EGF-, but also for serum- and LPA-induced DNA synthesis, suggesting that c-Src has the added capability of potentiating signals emanating from G-protein coupled receptors. Other reports have demonstrated a requirement for c-Src in signaling not only from EGF (47) and heterotrimeric G proteins (22, 23), but also from PDGF (35, 45), CSF-1, and integrin (39, 40) receptors. These studies suggest a necessity for c-Src in mitogenic and tumorigenic signaling from a number of different transmembrane receptors.

Previous attempts to understand the function of c-Src in cell growth and

tumorigenesis have relied heavily on mutational analysis of the different domains of c-Src and a determination of the functional effects of these mutations in model cell systems. c-Src is composed of several functional domains, which are grouped into the N-terminal regulatory and C-terminal catalytic halves of the molecule. The amino-terminal (N-Term) half of c-Src can be divided into several distinct domains. Sequences necessary for myristylation and membrane association are conserved among family members and between species and are contained within the first 17 amino acids (8, 15). The amino acid sequences immediately downstream of the membrane association domain are not conserved among the Src family of tyrosine kinases, generating the diversity typical of this region, termed the Unique domain. The function of c-Src's Unique domain is not well-defined, but it contains mitosis-specific sites of phosphorylation (43) and has been reported to bind a number of different signaling molecules (32). The remaining portion of the N-term contains two protein-protein interaction domains, the SH2 and SH3 domains, which bind phospho-tyrosine or polyproline-containing motifs, respectively, in a variety of signaling proteins (30). Among these proteins are FAK (38) and p130CAS (26), two proteins involved in cell adhesion and migration. In fact, the N-term of c-Src, containing intact SH2 and SH3 domains, can functionally replace full length *wt* c-Src in the proper formation of focal adhesions and in the ability of a cell to spread on fibronectin (16, 17), suggesting that these domains may be involved in the regulation of transformed cell morphologies.

The catalytic domain is contained within the carboxy-terminal (C-Term) half of the protein. The kinase activity of c-Src is negatively regulated by intramolecular interactions between phosphorylated Tyr-527 in the extreme C-terminal regulatory domain and its own

SH2 domain, and between the SH2-kinase linker and its own SH3 domain (20, 36, 48). Mutations that disrupt either of these interactions, including Tyr to Phe alterations at position 527 or disrupting the SH2 or SH3 domains, results in an activation of the kinase and transformation of the cell (4, 19, 31, 33, 34, 41).

The prevalence of c-Src in human cancers and in a variety of signal transduction pathways suggests that it may be required for tumor development or maintenance and thus may be a useful target in cancer therapy. Using a model system consisting of several breast tumor cell lines and pharmacological inhibitors or molecularly altered variants of c-Src, we show here that c-Src is required for maintenance of the tumorigenic phenotype as measured both *in vitro* and in vivo. In an effort to further understand c-Src's role in tumorigenesis and to define domains that mediate growth inhibition in an initial attempt to therapeutically target these domains, we expressed isolated domains of c-Src in a breast tumor cell line, MDA-MB-468. Surprisingly, the C-term, independent of its kinase activity, and to a lesser degree the SH2 domain, were able to inhibit soft agar growth and BrdU incorporation in serum-containing media. Further analysis revealed specific and stable binding of an approximately 95 kDa protein to the C-term of c-Src, suggesting that this region may have a protein-protein interaction function in addition to catalytic activity.

Materials and Methods

Constructs. A pcDNA3 vector (Invitrogen, Carlsbad, CA) encoding wt chicken c-Src was constructed by inserting a HindIII-EcoRI fragment containing the wt c-Src cDNA (from pRL plasmid, gift of J.T. Parsons, University of Virginia) into the corresponding HindIII-EcoRI site of the pcDNA3 vector. Kinase-inactive (K-) c-Src was isolated by PCR from pm430 plasmid (47) and cloned into the HindIII-EcoRI site of the

pcDNA3 vector. Individual c-Src domains were generated by PCR from either *wt* or K- (for C-term) c-Src pcDNA3 vector templates and cloned into the XbaI-HindIII site of the pcDNA3.1 myc-tag vector (Invitrogen, Carlsbad, CA). Specifically, sequences encoding the Unique domain (residues 1-84), the SH3 domain (residues 85-149), the SH2 domain (residues 150-260), the N-term (residues 1-260) and the C-term (residues 261-533) of the chicken c-Src protein were isolated. The first 7 amino acids of c-Src were added to the amino terminus of the isolated SH3 and SH2 domains during the PCR reaction. The sequences of each construct were confirmed by automated DNA sequence analysis. K- c-Src and K- C-term were confirmed to be kinase-inactive by *in vitro* kinase asssay (data not shown).

Cell Lines. Maintenance of the breast tumor cell lines in culture has been described previously (2). MDA-MB-468 and MCF-7 cell lines, stably expressing either K- c-Src or c-Src domains, were derived by transfection of appropriate plasmids with Lipofectin (GibcoBRL, Gathersburg MD), selection by G418 resistance, and cloning by limiting dilution. Clonal populations were screened for expression of the construct by Western immunoblotting. Fold overexpression of the domains was estimated by comparative Western blotting analysis with endogenous c-Src using a c-Src-specific antibody directed against amino acids 2-17.

Western Immunoblotting. Western blot analysis was performed as previously described (24, 47), using purified 2-17 mouse monoclonal antibody (mAb) (Quality Biological, Inc., Gaithersburg MD) to identify c-Src, and anti-myc tag mAb (Zymed, San Francisco, CA) to identify the myc-tagged c-Src domains. [^{125}I]-protein A (ICN, Costa Mesa CA) or [^{125}I]-labeled goat anti-mouse immunoglobulin (New England Nuclear,

Boston, MA) and autoradiography were employed to localize binding of primary antibodies.

Colony Formation in Soft Agar and Tumorigenicity. Anchorage-independent growth was measured as previously described (24). The indicated number of cells were plated in 60 mm dishes for each of the breast tumor cell lines. PP1 (Calbiochem, San Diego CA) was used at a final concentration of 10 μM and supplemented every 2-4 days. Two-three week old colonies were stained for 20 hr at 37 °C in a solution of iodonitrotetrazolium salt (1μg/ml; Sigma, St. Louis MO) in water and counted using EagleSight analysis software (Stratagene, La Jolla CA). The soft agar colony data for the MDA-MB-468 clones expressing various c-Src domains include analysis of two separate clones for each cell type; (468 U_6, 468 U_8), (468 3_{15}, 468 3_{18}), (468 2_{11}, 468 2_{21}), (468 N_4, 468 N_7), and the 468 C-term as indicated. Assessment of tumor formation in Taconic nu/nu mice was performed as previously described (24).

Transient Transfections and BrdU Incorporation. A 50-70 % confluent, 35 mm dish of cells was transfected with 20 μl Lipofectin and 4 μg plasmid DNA according to manufacturer's directions and incubated in a humidified, 37^0C, 5% CO_2 atmosphere for 24 hr. Transfected cells were allowed to recover overnight in growth medium containing 10% FBS, after which they were incubated with 100 μM BrdU for 17 hr and co-stained for myc-tag expression and BrdU incorporation as described by the manufacturer of the BrdU-specific mAb (Boehringer-Mannheim, Indianapolis IN). Specifically, fixed cells were treated with 2N HCl for 1 hr at 37°C and incubated with a 1:500 dilution of rabbit polyclonal anti-myc tag antibody (residues 409-420)(Upstate Biotechnology, Lake Placid NY) followed by incubation with a mixture of antibodies (1:1000 dilution of texas red-

conjugated goat anti-rabbit IgG from Jackson Immunoresearch Laboratories, West Grove, PA, and a 1:15 dilution of FITC-conjugated anti-BrdU mAb from Boeringer Mannheim, Indianapolis, IN).

Immunofluorescence. Cos-7 cells were transiently transfected with plasmids encoding the various c-Src constructs using Lipofectin as described above. The next day, cells were fixed and stained for expression as previously described (5), with either 1:1000 rabbit polyclonal myc-tag antibody to visualize the c-Src domains or 1:5000 EC10 mouse mAb (29) to visualize either full-length *wt* or K- c-Src. This was followed by incubation with either 1:1000 dilution of texas red-conjugated goat anti-rabbit IgG or FITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA). For co-localization analysis, antibodies were used in combination.

[^{35}S] Metabolic Labeling. Clonal cell lines of MDA-MB468 cells expressing the various domains of c-Src were starved for 1 hr in DMEM without L-methionine (Gibco BRL), but containing 5% dialyzed FBS. 125 μCi/ml of [^{35}S]-L-methionine (Sp. Act. = 1175 Ci/mmol; New England Nuclear) was added, and cultures were incubated overnight in a humidified, 37°C, 5% CO_2 atmosphere. Extracts were immunoprecipitated with 5 μg rabbit polyclonal anti-myc tag antibody or 5 μg of rabbit anti-mouse antibody (Jackson Immunoresearch Laboratories) and separated by SDS-PAGE.

Results

c-Src is required for breast tumor cell tumorigenicity. C-Src is overexpressed 5 fold or greater above normal in MDA-MB468, MCF-7, and ZR-75-1 breast tumor cell lines (2). To assess the requirement for c-Src or its family members in the growth of these breast carcinoma cells, we treated the cells with a Src family inhibitor, PP1 (12), and

measured their ability to grow in soft agar. Treatment with PP1 dramatically inhibited colony formation of all three tumor cell lines (Fig. 1), suggesting a requirement for c-Src or related family members in the ability of these cells to grow in anchorage independent conditions. Treatment of three additional breast tumor cell lines with PP1 (UACC-812, MDA-MB-361, and MDA-MB-453) also resulted in an inhibition of soft agar growth (Belsches-Jablonski *et al.*, submitted).

As a separate measure of c-Src's requirement for tumorigenicity in these cells, kinase-inactive (K-) c-Src was stably transfected into two different breast tumor cell lines, MDA-MB468 and MCF-7 cells. Two clones in each cell line that expressed different amounts of K- c-Src were chosen for further analysis (Fig. 2A). K- c-Src inhibited soft agar colony formation in a dose-dependent fashion when expressed in both breast tumor cell types (Fig. 2B). This dose-dependent inhibition of MDA-MB468 soft agar growth by K- c-Src was replicated *in vivo* in a tumor formation study in nude mice (Table I), demonstrating for the first time a requirement for c-Src in the maintenance of the tumorigenic phenotype of these cells. These studies also support the hypothesis that c-Src kinase activity is an effective therapeutic target in breast cancer.

The C-terminus (C-term) and SH2 domain of c-Src inhibit growth of MDA-MB-468 breast tumor cells. To determine which domain of c-Src was responsible for the inhibition of tumorigenicity in the breast tumor cells, we generated carboxy-terminally myc-tagged constructs of each domain or combination of domains (Fig. 3). The isolated SH2 and SH3 domains included an amino-terminal myristylation signal to assist in proper localization, since the isolated SH3 domain lacking a myristylation sequence was found to localize almost exclusively to the nucleus (data not shown). Stable clones of MDA-

MB468 cells expressing each of the indicated constructs were generated and screened for levels of expression (Fig. 4A). All of the domains were overexpressed 3-20 fold above endogenous levels of c-Src, except the C-term, which most frequently was observed at levels comparable to endogenous (data not shown).

To determine which, if any, of the isolated c-Src domains inhibited anchorage independent growth in a manner similar to K- c-Src, two MDA-MB468 clones of each domain were tested for their ability to form colonies in soft agar. Two domains were found to significantly reduce colony formation as compared to the vector-only control, the C-term and the SH2 domain. Interestingly, two clones expressing the C-term inhibited soft agar growth more potently than the other domains (Fig. 4B). C-term$_{12}$, the only clone isolated that stably expressed the domain above endogenous levels (Fig. 4A), exhibited the most striking inhibition of soft agar growth. Clones C-term$_5$ and C-term$_{13}$, which expressed the domain at lower levels, less than or equal to endogenous c-Src, gave more variable results. Clonal variability was observed in four other clones expressing the C-term domain at low levels when assayed for growth in soft agar, with half of the clones showing >50% inhibition (data not shown), suggesting the net effect of the C-term is in fact an inhibition of soft agar growth. These results suggest a dose-dependency of inhibition and a high potency of inhibition by the C-term as compared to the other domains. The SH2 domain also showed significant inhibition of soft agar growth, however the effect was somewhat diminished when the SH2 domain was expressed in the context of the entire amino-terminus (N-term) (Fig. 4B). The SH3 domain also effected a modest, but significant inhibition of growth.

Since we had difficulty detecting significant overexpression of the C-term in stable clones and observed functional clonal variability in low expressors, we wanted to further investigate the inhibitory effect of this domain on the growth of MDA-MB468 cells. To this end, MDA-MB468 cells were transiently transfected with the various c-Src domains, allowed to recover for two days, and then assayed for their ability to undergo DNA synthesis by measuring BrdU incorporation under normal, serum-augmented growth conditions. Results from the BrdU incorporation assay were similar to those obtained from the soft agar assay, with the C-term exhibiting the most effective inhibition of all the domains at 50% (Fig. 5). This result suggests that the low expression and clonal variability observed with the C-term expressing clones may be due in part to the strong inhibition of DNA synthesis of cells grown in the presence of serum. The SH2 domain also exhibited partial inhibition of BrdU incorporation, with its effects once again being diminished in the presence of the entire N-term. The SH3 domain, however, exerted no inhibitory effect in this assay, suggesting that it influences predominantly anchorage-independent growth. The inability of any of the clones to undergo DNA synthesis did not appear to be due to the induction of apoptosis as assessed by a TUNEL assay 48 hours post-transfection (data not shown).

Co-localization of full length K- c-Src and the C-term. To determine the intracellular localization of the C-term in the cell, we transiently transfected COS cells with plasmids encoding full-length c-Src or each of the Myc-tagged constructs, and analyzed the transfected cells by immunofluorescent microscopy. COS cells were used to demonstrate localization of the domains because MDA-MB468 cells have a severely rounded morphology and little visible cytoplasm. Wt c-Src has previously been shown to

localize to perinuclear regions around microtubule organizing centers, late endosomes, and under certain conditions to focal adhesions (9, 18). Less abundant amounts can also be seen at the cell periphery. These same features were evident in COS cells expressing *wt* and K- c-Src (Fig. 6, panels A & B). The Unique, SH3, SH2 and N-term domains, which contained the myristylation sequences known to be important for proper targeting of c-Src, all displayed a diffuse cytoplasmic staining along with strong perinuclear staining (Fig. 6, panels E-H). The Unique, SH2 and N-term also showed staining at the cell edge. The C-term of c-Src, which lacked the amino-terminal myristylation domain, exhibited a diffuse cytoplasmic staining along with punctate perinuclear staining (Fig. 6, panel C). The punctate perinuclear staining was similar to the previously identified late endosomal staining observed in cells expressing the full-length *wt* c-Src molecule (18).

To further determine if the C-term localized to the same structures as the full-length molecule, we expressed full-length K- c-Src and the kinase-inactive C-term together in COS cells. Co-localization was observed by using EC10 mouse mAb, directed against the Unique domain of chicken c-Src, to recognize K- c-Src and a rabbit polyclonal myc-tag antibody to recognize the C-term, followed by different fluorescent-conjugated secondary antibodies. The C-term, lacking the myristylation domain, co-localized with full-length K- c-Src in distinct perinuclear regions (Fig. 6, panel D), suggesting that the C-term of c-Src contains sequences that target the molecule to the endosomal-like structures. This result also suggests that the C-term and K- c-Src may be exhibiting their inhibitory effects in these structures.

Co-precipitation of a ~95kDa protein with the C-term. The C-term of c-Src, containing only the kinase domain and the negative regulatory domain, has not been

shown to be involved in subcellular targeting of c-Src or in stable interactions with other proteins. Because of its dominant-negative effects on tumor cell growth, we sought to identify potential binding partners of the C-term. Therefore, we immunoprecipitated the various domains of c-Src from [^{35}S]-methionine metabolically labeled, stably transfected clones of MDA-MB468 breast tumor cells. We found an approximately 95kDa polypeptide co-precipitating specifically with the C-term of c-Src in two separate clones (Fig. 7). The specific presence of this band was observed in multiple experiments (data not shown). These results suggest that the C-term of c-Src is sufficient for binding to a protein of ~95kDa. Surprisingly, the isolated SH2 and SH3 domains did not consistently co-precipitate any specific bands.

Discussion

Using several breast tumor cell lines as a model, we show that c-Src is required for tumorigenicity of these cells both *in vitro* and in vivo. We further demonstrate that the C-term, independent of its kinase activity, and to a lesser degree the SH2 domain, are able to inhibit soft agar growth and BrdU incorporation. The C-term was found to co-localize with full-length K- c-Src in endosomal-like structures and to specifically bind to a protein of approximately 95 kDa, further suggesting that it may have a protein interaction function in addition to catalytic activity. These results suggest that disruption of C-term interactions may be an effective therapy for breast cancer.

The specific role of c-Src in tumor formation is not yet clearly understood. Overexpression in a number of different human tumor types suggests that it may be an important progression factor or other mediator of a tumorigenic event. This report demonstrates for the first time, that c-Src is required for tumorigenesis of several breast

tumor cell lines. This is accomplished by two independent methods of inhibition, using both pharmacological agents and cellular expression of dominant negative forms of c-Src. While both methods individually can be questioned as to their specificity, together they strongly argue for a requirement for c-Src family members in tumorigenesis of these cells. Since overexpression of c-Src alone is insufficient to cause transformation, c-Src's role may be one of a maintenance or progression factor for tumor development. c-Src may be acting together with other oncogenes, such as growth factor receptors, or coordinately with other tumorigenic factors to give rise to a more aggressive tumorigenic phenotype. Nevertheless, these results implicate the targeting of c-Src as a useful approach to inhibt cancer.

With this therapeutic angle in mind, we attempted to dissect the inhibition of K- c-Src in breast tumor cells. We reasoned that a potential mechanism of inhibition may be that K- c-Src is sequestering critical signal transducing molecules and not allowing other components of pathways to signal through them. If this were the case, then it might be predicted that one or more of the domains may be responsible for binding critical factors. The two protein-protein interaction domains, the SH2 and SH3 domains, in the N-term of c-Src are prime candidates for such saturating domains. Several proteins are known to bind to the c-Src SH2 and SH3 domains including FAK (38), p130CAS (26), dynamin (10), and growth factor receptors (27). The SH2 domain was partially inhibitory in both the soft agar assays and the BrdU assays. However, it was interesting to note that in both assays the magnitude of inhibition was decreased when the SH2 domain was in the context of the entire N-term. This may reflect either the added specificity, or the direct interference of other domains in an entire N-term compared to an isolated SH2 domain. It

may be that the SH2 domain is a more promiscuous P-Tyr binding domain in the absence of the other domains and therefore is able to sequester more signaling molecules. The SH3 and Unique domains had little to no effect on either growth assay, suggesting that neither is sufficient for inhibition. Both also serve as important negative controls for the effects of the C-term and SH2 domains on growth.

The entire N-term of c-Src is known to bind to normal cellular partners of c-Src, such as p130CAS (39), and be sufficient for reconstituting focal adhesion structures and cell spreading (16, 17). We originally hypothesized that this domain would act identically to K- c-Src and might inhibit tumorigenicity of the breast tumor cells by allowing signaling from integrins in the focal adhesions, thus restoring anchorage dependent growth conditions. Surprisingly, this was not the case, and instead, the N-term served to diminish the inhibitory effects of the isolated SH2 domain. This suggested that the C-term of c-Src may be sufficient to mediate the inhibition seen with the full-length molecule.

The C-term of c-Src is composed of a tyrosine kinase domain and a 17aa negative regulatory domain. The myristylation domain was not added to the amino-terminus of the C-term, which therefore lacked any known targeting sequences. However, we observed co-localization of the C-term with full length K- c-Src in distinct perinuclear regions. The punctate staining pattern observed was similar to the intracellular vesicle/late endosomal localization previously described for *wt* c-Src (9, 18, 28). This co-localization is significant because it demonstrates an inherent ability of the C-term to be targeted properly to endosomal-like structures, and suggests that the C-term and K- c-Src may be inhibiting tumorigenicity by a similar mechanism, perhaps involving regulation of endosomal trafficking. This hypothesis is supported by the findings that c-Src is localized

to endosomes and has been previously suggested to be involved in endocytosis and internalization of cell surface receptors (10, 46). The exact mechanism of regulation of endocytic events by c-Src is unknown.

The C-term lacked catalytic activity and thus did not require tyrosine kinase activity to mediate the inhibition. Therefore, it may be interfering with tumorigenic signaling by interacting with essential molecules. The two domains of the C-term are each reasonable candidates for binding cellular proteins. The negative regulatory domain contains a Tyr at position 527 that is known to be phosphorylated by CSK (14). Without the presence of the N-terminal SH2 domain to which it normally binds, this P-Tyr could potentially bind other SH2 or PTB domain-containing proteins. However, the C-term does not appear to be phosphorylated on Tyr as can be seen by Western blotting with P-Tyr antibodies (data not shown). The negative regulatory domain has been shown to be required for binding to polyomavirus middle T antigen independently of phosphorylation of Tyr527 (4), suggesting that this short peptide sequence may still be important for binding.

The kinase domain itself is also a candidate for a protein interaction domain. The idea that sequences in a kinase domain can bind to other molecules independent of its kinase activity is becoming more prevalent. In fact, the isolated kinase domain of c-Src has been shown previously to be capable of binding the EGFR (37). The kinase domain of a c-Src family member, c-Fyn, can also bind to a transmembrane receptor in hematopoietic cells, the erythropoietin receptor (6). The Janus family kinases (JAKs) have been found to bind to a number of signaling molecules through their active kinase domains (1, 11, 25). Still other reports demonstrate binding of receptor tyrosine kinase domains to scaffolding

proteins important in vesicle transport, such as caveolin (7) and beta-coatamer (13). These reports support the hypothesis that a kinase domain can have functions other than catalytic activity, as we observe with the kinase-inactive C-term of c-Src in binding to a ~95kDa protein. We have attempted to identify this protein by Western blotting using antibodies specific for various signaling molecules, and thus have shown that p95 is not STAT1, STAT3 or Hsp90 (data not shown). We are currently exploring other options and methods of identification.

In summary, this study provides evidence to warrant further development of methods to inhibit c-Src in cancer. It is very likely that c-Src is involved in the genesis or maintenance of other types of human tumors, given the large number of tumor types in which it is overexpressed. In this regard, we are interested in further exploring the mechanism of inhibition of tumorigenicity by K- c-Src and the C-term with hopes that we will be able to more specifically interdict c-Src-mediated signaling in human cancers.

Acknowledgments

We wish to thank Steve Gibson for technical assistance in the generation of the myc-tagged constructs and the members of the Parsons-Weber-Parsons group for critical evaluation of this work. This work was supported by DHHS grants CA3948 and CA71449 (SJP), CTR grant 4621 (SJP), and DOD grant DAMD 17-96-1-6126 (DAT).

Reference List

1. **Barahmand-Pour, F., A. Meinke, B. Groner, and T. Decker**. 1998. Jak2-Stat5 interactions analyzed in yeast. J.Biol.Chem. **273**:12567-12575.

2. **Biscardi, J.S., A.P. Belsches, and S.J. Parsons**. 1998. Characterization of human

epidermal growth factor receptor and c-Src interactions in human breast tumor cells. Mol.Carcinog. **21**:261-272.

3. **Biscardi, J.S., M.C. Maa, D.A. Tice, M.E. Cox, T.H. Leu, and S.J. Parsons.** 1999. c-Src-mediated phosphorylation of the EGF receptor on TYR 845 and TYR 1101 is associated with modulation of receptor function. J.Biol.Chem. **274**:8335-8343.

4. **Cartwright, C.A., W. Eckhart, S. Simon, and P.L. Kaplan.** 1987. Cell transformation by pp60c-src mutated in the carboxy-terminal regulatory domain. Cell **49**:83-91.

5. **Chang, J.H., S. Gill, J. Settleman, and S.J. Parsons.** 1995. c-Src Regulates the Simultaneous Rearrangement of Actin Cytoskeleton, p190RhoGAP, and p120RasGAP Following EGF Stimulation. J.Cell Biol. **130**:1-14.

6. **Chin, H., A. Arai, H. Wakao, R. Kamiyama, N. Miyasaka, and O. Miura.** 1998. Lyn physically associates with the erythropoietin receptor and may play a role in activation of the Stat5 pathway. Blood **91**:3734-3745.

7. **Couet, J., M. Sargiacomo, and M.P. Lisanti.** 1997. Interaction of a Receptor Tyrosine Kinase, EGF-R, with Caveolins. J.Biol.Chem. **272**:30429-30438.

8. **Cross, F.R., E.A. Garber, D. Pellman, and H. Hanafusa.** 1984. A short sequence

in the p60src N terminus is required for p60src myristylation and membrane association and for cell transformation. Mol.Cell.Biol. **4**:1834-1842.

9. **David-Pfeuty, T. and Y. Nouvian-Dooghe**. 1990. Immunolocalization of the cellular src protein in interphase and mitotic NIH c-src overexpresser cells. J.Cell Biol. **111**:3097-3116.

10. **Gout, I., R. Dhand, I.D. Hiles, M.J. Fry, G. Panayotou, P. Das, O. Truong, N.F. Totty, J. Hsuan, G.W. Booker, and a. et**. 1993. The GTPase dynamin binds to and is activated by a subset of SH3 domains. Cell **75**:25-36.

11. **Gual, P., V. Baron, V. Lequoy, and E. Van Obberghen**. 1998. Interaction of Janus kinases JAK-1 and JAK-2 with the insulin receptor and the insulin-like growth factor-1 receptor. Endocrinology **139**:884-893.

12. **Hanke, J.H., J.P. Gardner, R.L. Dow, P.S. Changelian, W.H. Brissette, Weringer, EJ, B.A. Pollok, and P.A. Connelly**. 1996. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. J.Biol.Chem. **271**:695-701.

13. **Hansen, L.A., N. Alexander, M.E. Hogan, J.P. Sundberg, A. Dlugosz, D.W. Threadgill, T. Magnuson, and S.H. Yuspa**. 1997. Genetically null mice reveal a central role for epidermal growth factor receptor in the differentiation of the hair follicle and normal hair development. Am.J.Pathol. **150**:1959-1975.

14. **Imamoto, A. and P. Soriano**. 1993. Disruption of the csk gene, encoding a negative regulator of Src family tyrosine kinases, leads to neural tube defects and embryonic lethality in mice. Cell **73**:1117-1124.

15. **Kaplan, J.M., G. Mardon, J.M. Bishop, and H.E. Varmus**. 1988. The first seven amino acids encoded by the v-*src* oncogene act as a myristylation signal: lysine 7 is a critical determinant. Mol.Cell.Biol. **8**:2435-2441.

16. **Kaplan, K.B., K.B. Bibbins, J.R. Swedlow, M. Arnaud, D.O. Morgan, and H.E. Varmus**. 1994. Association of the amino-terminal half of c-Src with focal adhesions alters their properties and is regulated by phosphorylation of tyrosine 527. EMBO J. **13**:4745-4756.

17. **Kaplan, K.B., J.R. Swedlow, D.O. Morgan, and H.E. Varmus**. 1995. c-Src enhances the spreading of src-/- fibroblasts on fibronectin by a kinase-independent mechanism. Genes Dev. **9**:1505-1517.

18. **Kaplan, K.B., J.R. Swedlow, H.E. Varmus, and D.O. Morgan**. 1992. Association of p60c-src with endosomal membranes in mammalian fibroblasts. J.Cell Biol. **118**:321-333.

19. **Kmiecik, T.E. and D. Shalloway** . 1987. Activation and suppression of pp60c-src transforming ability by mutation of its primary sites of tyrosine phosphorylation. Cell **49**:65-73.

20. **Liu, X., S.R. Brodeur, G. Gish, Z. Songyang, L.C. Cantley, A.P. Laudano, and T. Pawson**. 1993. Regulation of c-Src tyrosine kinase activity by the Src SH2 domain. Oncogene **8**:1119-1126.

21. **Luttrell, D.K., L.M. Luttrell, and S.J. Parsons**. 1988. Pp60c-Src tyrosine kinase, myristylation, and modulatory domains are required for enhanced mitogenic responsiveness to epidermal growth factor seen in cells overexpressing c-src. Mol.Cell.Biol. **8**:497-501.

22. **Luttrell, L.M., R.G. Della, B.T. van, D.K. Luttrell, and R.J. Lefkowitz**. 1997. Gbetagamma subunits mediate Src-dependent phosphorylation of the epidermal growth factor receptor. A scaffold for G protein-coupled receptor-mediated Ras activation. J.Biol.Chem. **272**:4637-4644.

23. **Luttrell, L.M., B.E. Hawes, B.T. van, D.K. Luttrell, T.J. Lansing, and R.J. Lefkowitz**. 1996. Role of c-Src tyrosine kinase in G protein-coupled receptor- and Gbetagamma subunit-mediated activation of mitogen-activated protein kinases. J.Biol.Chem. **271**:19443-19450.

24. **Maa, M.C., T.H. Leu, D.J. McCarley, R.C. Schatzman, and S.J. Parsons**. 1995. Potentiation of EGF Receptor-mediated Oncogenesis by c-Src: Implications for the Etiology of Multiple Human Cancers. Proc.Natl.Acad.Sci.USA **92**:6981-6985.

25. **Masuhara, M., H. Sakamoto, A. Matsumoto, R. Suzuki, H. Yasukawa, K.**

Mitsui, T. Wakioka, S. Tanimura, A. Sasaki, H. Misawa, M. Yokouchi, M. Ohtsubo, and A. Yoshimura. 1997. Cloning and characterization of novel CIS family genes. Biochem.Biophys.Res.Commun. **239**:439-446.

26. **Nakamoto, T., R. Sakai, K. Ozawa, Y. Yazaki, and H. Hirai.** 1996. Direct binding of C-terminal region of p130Cas to SH2 and SH3 domains of Src kinase. J.Biol.Chem. **271**:8959-8965.

27. **Parsons, J.T. and S.J. Parsons** . 1997. Src family protein tyrosine kinases: cooperating with growth factor and adhesion signaling pathways. Curr.Opin.Cell Biol. **9**:187-192.

28. **Parsons, S.J. and C.E. Creutz.** 1986. p60c-src activity detected in the chromaffin granule membrane. Biochem.Biophys.Res.Commun. **134**:736-742.

29. **Parsons, S.J., D.J. McCarley, C.M. Ely, D.C. Benjamin, and J.T. Parsons.** 1984. Monoclonal antibodies to Rous sarcoma virus pp60src react with enzymatically active cellular pp60src of avian and mammalian origin. J.Virol. **51**:272-282.

30. **Pawson, T. and J. Schlessinger** . 1993. SH2 and SH3 domains. Curr.Biol. **3**:434-442.

31. **Piwnica-Worms, H., K.B. Saunders, T.M. Roberts, A.E. Smith, and S.H.**

Cheng. 1987. Tyrosine phosphorylation regulates the biochemical and biological properties of pp60c-src. Cell **49**:75-82.

32. **Pleiman, C.M., M.R. Clark, L.K. Gauen, S. Winitz, K.M. Coggeshall, G.L. Johnson, A.S. Shaw, and J.C. Cambier**. 1993. Mapping of sites on the Src family protein tyrosine kinases p55blk, p59fyn, and p56lyn which interact with the effector molecules phospholipase C-gamma 2, microtubule-associated protein kinase, GTPase-activating protein, and phosphatidylinositol 3-kinase. Mol.Cell.Biol. **13** :5877-5887.

33. **Potts, W.M., A.B. Reynolds, T.J. Lansing, and J.T. Parsons**. 1988. Activation of pp60c-src transforming potential by mutations altering the structure of an amino terminal domain containing residues 90-95. Oncogene Res. **3**:343-355.

34. **Reynolds, A.B., J. Vila, T.J. Lansing, W.M. Potts, M.J. Weber, and J.T. Parsons**. 1987. Activation of the oncogenic potential of the avian cellular src protein by specific structural alteration of the carboxy terminus. EMBO J. **6**:2359-2364.

35. **Roche, S., M. Koegl, M.V. Barone, M.F. Roussel, and S.A. Courtneidge**. 1995. DNA synthesis induced by some but not all growth factors requires Src family protein tyrosine kinases. Mol.Cell.Biol. **15**:1102-1109.

36. **Roussel, R.R., S.R. Brodeur, D. Shalloway, and A.P. Laudano**. 1991. Selective binding of activated pp60c-src by an immobilized synthetic phosphopeptide modeled

on the carboxyl terminus of pp60c-src. Proc.Natl.Acad.Sci.USA **88**:10696-10700.

37. **Sato, K.I., A. Sato, M. Aoto, and Y. Fukami**. 1995. c-Src phosphorylates epidermal growth factor receptor on tyrosine 845. Biochem.Biophys.Res.Commun. **215**:1078-1087.

38. **Schaller, M.D., J.D. Hildebrand, J.D. Shannon, J.W. Fox, R.R. Vines, and J.T. Parsons**. 1994. Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. J.Virol. **14**:1680-1688.

39. **Schlaepfer, D.D., M.A. Broome, and T. Hunter**. 1997. Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, p130cas, and Nck adaptor proteins. J.Virol. **17**:1702-1713.

40. **Schlaepfer, D.D. and T. Hunter** . 1997. Focal adhesion kinase overexpression enhances ras-dependent integrin signaling to ERK2/mitogen-activated protein kinase through interactions with and activation of c-Src. J.Biol.Chem. **272**:13189-13195.

41. **Seidel-Dugan, C., B.E. Meyer, S.M. Thomas, and J.S. Brugge**. 1992. Effects of SH2 and SH3 deletions on the functional activities of wild-type and transforming variants of c-Src. Mol.Cell.Biol. **12**:1835-1845.

42. **Shalloway, D., P.M. Coussens, and P. Yaciuk**. 1984. Overexpression of the c-src protein does not induce transformation of NIH 3T3 cells. Proc.Natl.Acad.Sci.USA

81:7071-7075.

43. **Shenoy, S., I. Chackalaparampil, S. Bagrodia, P.H. Lin, and D. Shalloway**. 1992. Role of p34cdc2-mediated phosphorylations in two-step activation of pp60c-src during mitosis. Proc.Natl.Acad.Sci.USA **89**:7237-7241.

44. **Tice, D.A., J.S. Biscardi, A.L. Nickles, and S.J. Parsons**. 1999. Mechanism of biological synergy between epidermal growth factor receptor and cellular Src. Proc.Natl.Acad.Sci.USA **96**:1415-1420.

45. **Twamley-Stein, G.M., R. Pepperkok, W. Ansorge, and S.A. Courtneidge**. 1993. The Src family tyrosine kinases are required for platelet-derived growth factor-mediated signal transduction in NIH-3T3 cells. Proc.Natl.Acad.Sci.USA **90**:7696-7700.

46. **Ware, M.F., D.A. Tice, S.J. Parsons, and D.A. Lauffenburger**. 1997. Overexpression of c-src in fibroblasts enhances endocytic internalization of epidermal growth factor receptor. J.Biol.Chem. **272**:30185-30190.

47. **Wilson, L.K., D.K. Luttrell, J.T. Parsons, and S.J. Parsons**. 1989. pp60c-src Tyrosine Kinase, Myristylation, and Modulatory Domains Are Required for Enchanced Mitogenic Responsiveness to Epidermal Growth Factor Seen in Cells Overexpressing c-src. Mol.Cell.Biol. **9**:1536-1544.

48. **Yamaguchi, H. and W.A. Hendrickson.** 1996. Structural basis for activation of human lymphocyte kinase Lck upon tyrosine phosphorylation. Nature **384**:484-489.

Figure Legends

Figure 1. PP1 inhibits soft agar colony formation of several breast tumor cell lines. Values for number of colonies are the mean ± SEM of at least 3 experiments in which 10^5 cells of each cell line were seeded per plate in triplicate and treated with either 10 μM PP1 or an equivalent volume of DMSO (filled bars) every 3-4 days.

Figure 2. Kinase-inactive c-Src dose-dependently inhibits soft agar colony formation of MDA-MB468 and MCF-7 breast tumor cells. A. Western immunoblot analysis of MDA-MB468 and MCF-7 clonal cell lines stably overexpressing kinase deficient (K-) c-Src. Fold overexpression was estimated by densitometry analysis. **B.** Values for number of colonies are the mean ± SEM of at least 3 experiments in which 10^5 cells of each clone were seeded per plate in triplicate.

Figure 3. Myc-tagged c-Src domain constructs. Various c-Src domains were PCR amplified as described in Materials and Methods and Results and cloned into a pcDNA3.1 myc epitope tagging vector.

Figure 4. The c-Src C-term and SH2 domain inhibit soft agar colony formation of MDA-MB468 breast tumor cells. Vector-only (black bar) and the indicated myc-tagged c-Src domains (open bars) were stably transfected into MDA-MB468 breast tumor cells, selected, and cloned as described in Materials and Methods. **A.** Western immunoblot analysis of clonal cell lines expressing myc-tagged c-Src domains. Arrow points to the C-

term domain. **B.** Values for number of colonies are the mean ± SEM of at least 3 experiments in which 10^5 cells of each clone were seeded per plate in triplicate. The unique, SH3, SH2 and N-term domains represent the values obtained from two independent clones expressing each domain. * P values < 0.05.

Figure 5. The c-Src C-term and SH2 domains inhibit BrdU incorporation in MDA-MB468 breast tumor cells. MDA-MB468 breast tumor cells were transfected with plasmid DNA encoding Myc-tagged c-Src domains, cultured for one day, and incubated with 100 μM BrdU for 17 hrs. Cells were fixed and co-stained for myc-tag expression and BrdU incorporation. Results are expressed as the mean percent ± SEM of cells expressing the myc-tag that were positive for BrdU incorporation. Approximately 100 cells were analyzed for each variable in 3 independent experiments. * P values ≤ 0.02.

Figure 6. Localization of c-Src and c-Src domains in transiently transfected COS cells. Cos cells were transiently transfected with plasmids encoding the indicated molecules, fixed and stained with either rabbit polyclonal myc-tag antibody or EC10 mouse mAb, or both for co-localization in panel D. Panels A, E, F, G, and H are shown at 400X while panels B, C, and D are shown at 1000X to demonstrate more detail.

Figure 7. Association of a ~95 kDa protein with the C-term of c-Src. MDA-MB468 clonal cell lines, C_5 and C_7 (C-term clone # 5 and # 7), U_6 (Unique # 6), 3_{15} (SH3 # 15), 2_{11} (SH2 # 11), and N_4 (N-term # 4), were metabolically labeled overnight with [^{35}S] methionine, and extracts were immunoprecipitated with rabbit polyclonal anti-myc antibody (+) or negative antibody control (-). Precipitated proteins were analyzed by SDS-PAGE and autoradiography. The band of ~30kDa in the C_7 and C_5 lanes represents the ectopically-expressed C-term of c-Src. Ectopically-expressed Unique, SH3, SH2, and N-

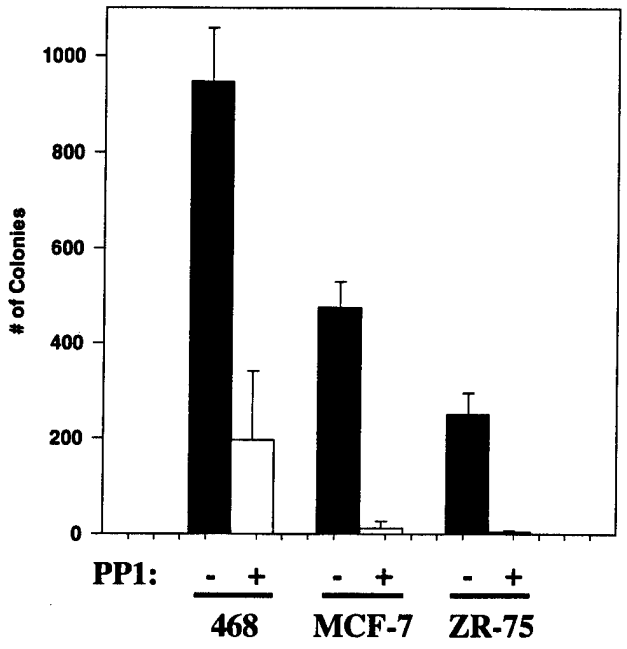
term domains were not observed in the autoradiogram due to the absence of methionines in the N-term of c-Src. However, the presence and nearly equal expression of these domains were confirmed by Western immunoblotting using rabbit polyclonal myc-tag antibody (data not shown).

TABLE 1. Kinase deficient c-Src dose-dependently inhibits tumor formation of MDA-MB-468 breast tumor cell line.

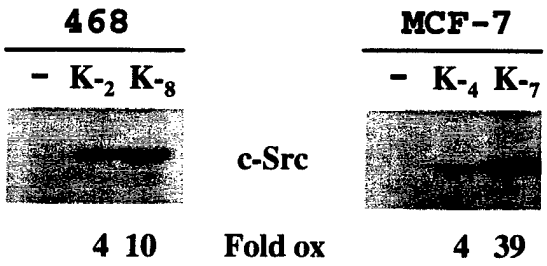
Cell line	Tumor volume (mm^3)*

MDA-MB-468	228 ± 45
468 K^-_2	102 ± 21
468 K^-_8	56 ± 8

* Mean tumor volume ± SEM of 8 individual sites was measured at day 23 after subcutaneous injection with 10^7 cells.


1000
800
600
400
200
0
# of Colonies
PP1:
- +
- +
- +
468
MCF-7
ZR-75

A.


468
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MCF-7
- K-4 K-7
c-Src
4 10
Fold ox
4 39

B.

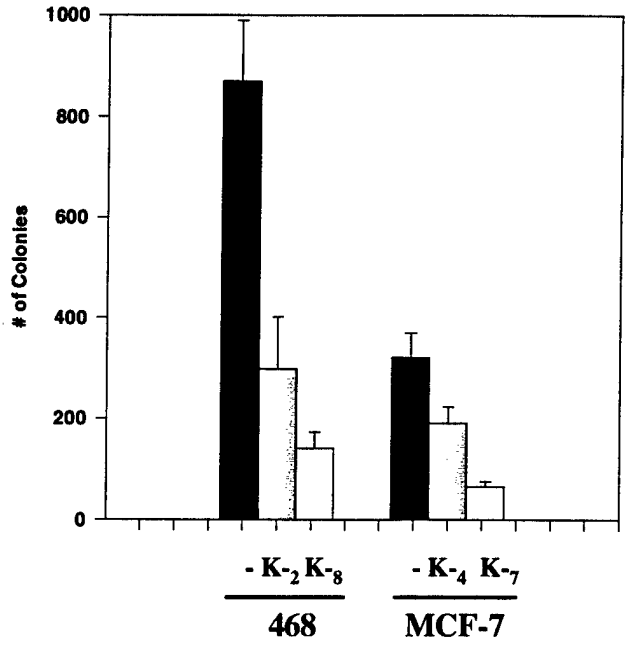
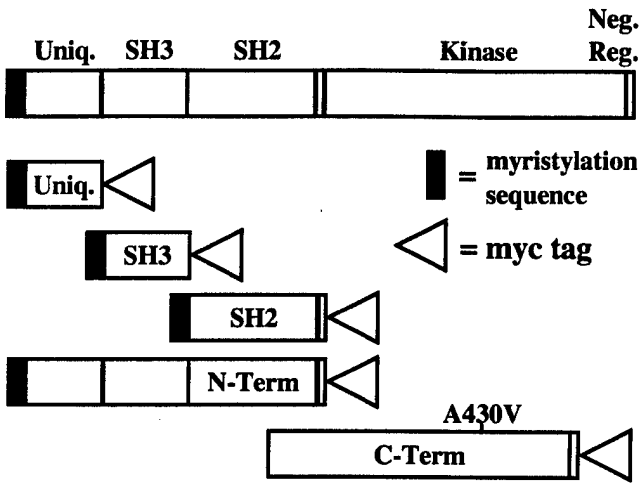

1000
800
600
400
200
0
# of Colonies
- K-2 K-8
- K-4 K-7
468
MCF-7

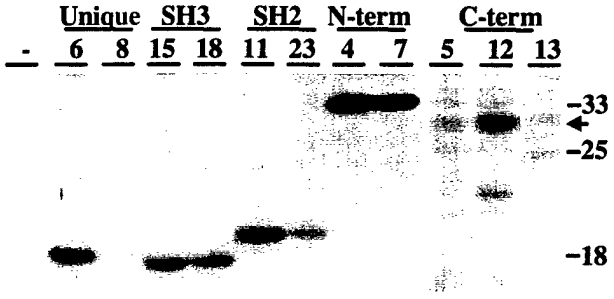
Table I. Kinase deficient c-Src inhibits tumor formation of MDA-MB-468 breast tumor cells in a dose-dependent manner.

Cell Line	Tumor Size*
MDA-MB-468	230 ± 40
468 K^-_2	100 ± 20
468 K^-_8	60 ± 10

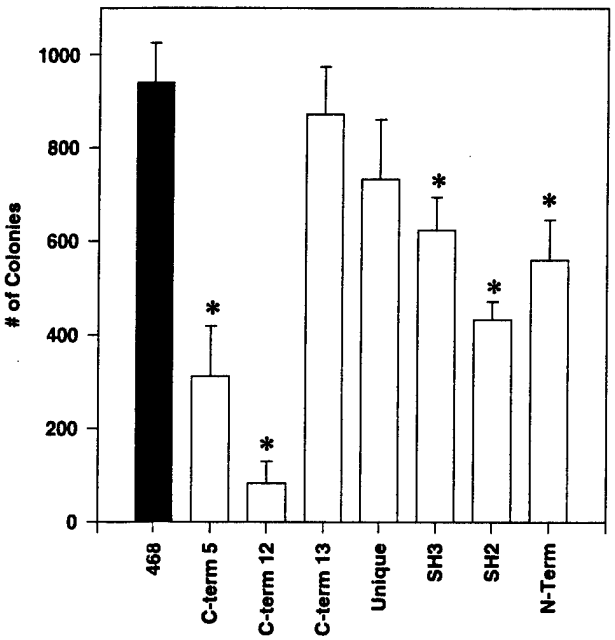
* Mean tumor volume ± SEM (expressed in mm^3) of 8 individual sites was measured at day 23 after subcutaneous injection of 10^7 cells into nu/nu mice.

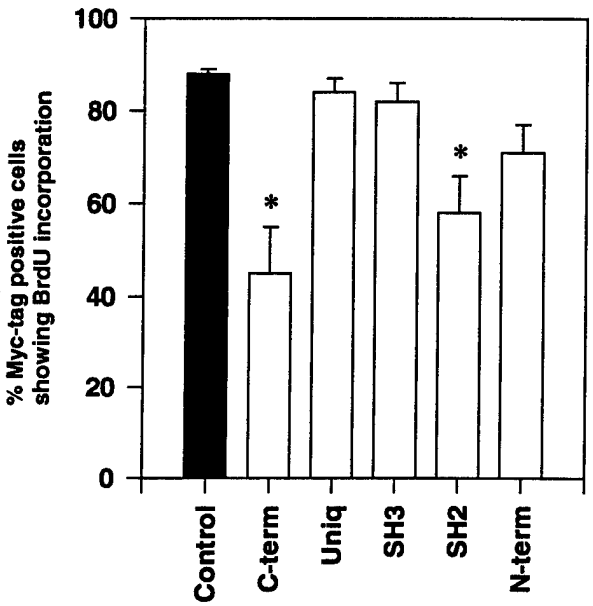

Uniq.
SH3
SH2
Kinase
Neg. Reg.
Uniq.
SH3
SH2
N-Term
A430V
C-Term
= myristylation sequence
= myc tag

A.


Unique
SH3
SH2
N-term
C-term
-
6
8
15
18
11
23
4
7
5
12
13
-33
-25
-18

B.


# of Colonies
1000
800
600
400
200
0
468
C-term 5
C-term 12
C-term 13
Unique
SH3
SH2
N-Term
*


% Myc-tag positive cells showing BrdU incorporation
100
80
60
40
20
0
*
*
Control
C-term
Uniq
SH3
SH2
N-term

A. *wt* Src

B. K- Src

C. C-term

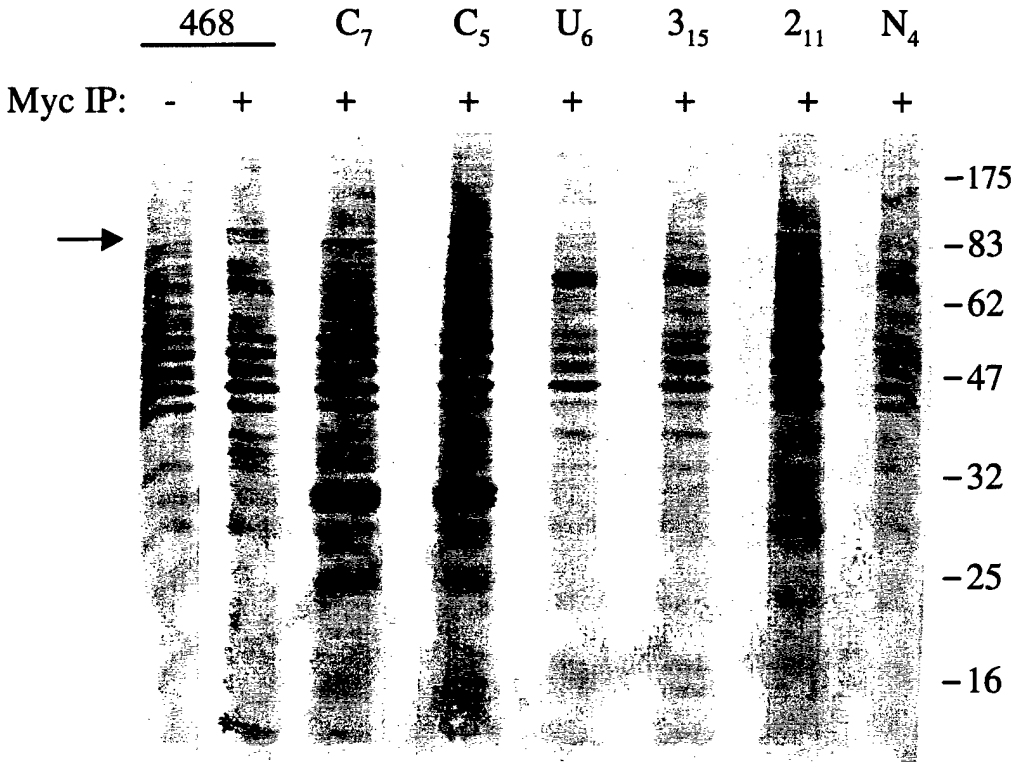
D. K- c-Src + C-term

E. Unique

F. SH3

G. SH2

H. N-Term


468
C7
C5
U6
315
211
N4
Myc IP: - + + + + + + +
-175
-83
-62
-47
-32
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